Development of the endodermal organs in Xenopus laevis

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Award date:
1999

Awarding institution:
University of Bath

DEVELOPMENT OF THE ENDODERMAL ORGANS IN *XENOPUS LAEVIS*

submitted by Andrew Douglas Chalmers

for the degree of PhD

of the University of Bath

1999

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ACKNOWLEDGEMENTS

First, I would like to warmly thank my supervisor Jonathan Slack who proposed this interesting project and provided me with excellent supervision and support throughout.

I would also like to acknowledge the Wellcome Trust who funded my studentship.

During this project I have been very fortunate to recieve a great deal of help and encouregement from the superb people in Jonathan's group. I would like to thank everybody who has helped me, including those who are still in the group and those that have now moved on.

Lastly and most importantly, I would like to thank my parents for getting me here, and Claire who for some reason puts up with me even when I am at my worst; I love you dearly.

SUMMARY

The epithelial lining of the gut, together with the pancreas, liver and gall bladder and the respiratory system is formed from the embryonic endoderm. The gut also contains smooth muscle and connective tissue of mesodermal origin. The amphibian *Xenopus laevis* is potentially an excellent model organism for studying the development of these organs. However, the anatomical complexity of the coiled gut presented a problem for studying its own development. In the first part of this study a comprehensive description of the anatomy, histology and gene expression of the developing endodermal organs is presented. This description made it possible to carry out the next part of this study, which was to produce an endoderm fate map. The endoderm fate map shows which parts of the endoderm give rise to which organs of the gut and respiratory system. A second fate map was also produced which shows the origin of the gut associated smooth muscle. Comparison of the two fate maps shows that for most organs of the gut the prospective epithelium and smooth muscle do not overlie each other in the early embryo, so must move into accord later in development. In the final part of this study a number of labelling techniques were used to investigate the morphogenesis of the gut epithelium from the embryonic endoderm. Radial intercalation was demonstrated to occur during epithelium formation and the embryonic archenteron cavity was shown to almost close before providing a nucleus for the opening of the definitive gut cavity. These results were combined to produce a model explaining the morphogenesis of the gut epithelium from the embryonic endoderm.

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LIST OF ABBREVIATIONS

abd-A	*abdominal-A*
alk-phos	alkaline phosphatase
Antp	*antennapedia*
Aph	anterior pharynx
ar-f	archenteron floor
ar-r	archenteron roof
bb	brush border
bd	bile duct
BSA	bovine serum albumin
ci	cilia
CNS	central nervous system
ct	connective tissue
DIG	*digoxigenin*
dpp	*decapentaplegic*
DTT	dithiothreitol
FDA	fluorescein dextran amine
FGF	fibroblast growth factor
gb	gall bladder
gi	gills
gp	gastric pits
HMG	high motility group
ht	heart
i	intestine
IFABP	intestinal fatty acid binding protein
IMS	industrial methylated spirits
IMZ	involuting marginal zone
l	gut lumen

lab	*labial*
li	large intestine
lic	internal coil of large intestine
lid	distal large intestine
lu	lungs
lv	liver
m	mesenchyme
MAB	maleic acid buffer
mRNA	messenger ribonucleic acid
ms	muscle
mu	mucus
n	number of cases
NAM	normal amphibian media
nd	nephritic duct
NIMZ	non involuting marginal zone
no	notochord
oe	oesophagus
ov	otic vesicle
pa	pancreas
PBS	phosphate buffered saline
PBST	phosphate buffered saline Tween 20
ph	pharynx
PMSG	pregnant mare serum gonadotropin
Pph	posterior pharynx
pr	proctodaeum
RNA	ribonucleic acid
RT-PCR	reverse transcriptase-polymerase chain reaction
si	small intestine
sia	proximal small intestine
sib	external coil of small intestine
sic	internal coil of small intestine
st	stomach

TGFβ	transforming growth factor beta
tn	tongue
tr	trachea
tz	*transitional zone*
Ubx	*ultrabithorax*
wg	*wingless*

CHAPTER 1 INTRODUCTION

1.1 INTRODUCTION TO THE ENDODERM

Early on in development all vertebrate embryos undergo a complex series of cell movements called gastrulation. Gastrulation divides the embryonic cells into 3 populations or germ layers (Fig. 1.1A). The outer layer is called the ectoderm, the middle layer the mesoderm and the inner layer the endoderm. The organisation of the three germ layers appears quite different in different vertebrates (for a description of vertebrate embryology see (Gilbert and Raunio, 1997). The germ layers of the post gastrulation chick embryos are flat oval discs floating on a "sea" of yolk. In contrast the germ layers of the amphibian *Xenopus laevis* are roughly spherical, arranged one on top of the other around a central cavity called the archenteron (Fig. 1.1A). Despite these topographic differences the germ layers of vertebrates show the same restrictions in cell fate. The ectoderm will form, for example, the central nervous system and the skin, while the mesoderm will form muscle, bone and blood of the adult. This work will focus on the cells of the endoderm which form the epithelial lining of the digestive tract and respiratory system.

1.2 THE DIGESTIVE AND RESPIRATORY SYSTEMS

The cells of the epithelia of the digestive tract, that is oesophagus, stomach, intestines and anus originate from the cells of the embryonic endoderm. The epithelia of the pancreas, liver and gall bladder which are associated with the digestive tract also originate from the cells of the endoderm. However, the organs of the digestive tract do not just contain an endoderm-derived epithelia but have a characteristic 4 layered structure (Fig. 1.1B, Wheater *et al.*, 1979). The four layers are;

1, the mucosa. The inner most layer of the digestive tract is the mucosa which can be sub-divided into a further three layers. The mucosa consists of an epithelium that

lines the gut lumen, supportive connective tissue called the lamina propria and a thin layer of smooth muscle known as the muscularis mucosae.

2, submucosa. The next layer of the digestive tract is the submucosa which lies outside the mucosa and consists of loose connective tissue.

3, muscularis. Surrounding the submucosa is the muscularis which is usually made up of two layers of smooth muscle.

4, adventitia. Finally, the outer most layer of the digestive tract is the connective tissue of the adventitia.

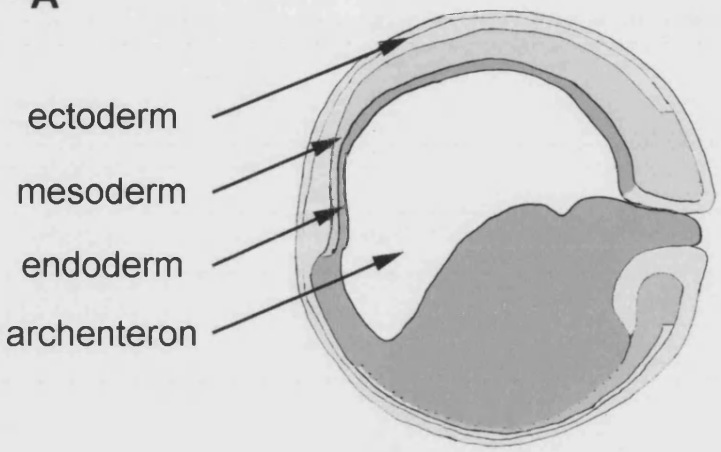
Although, each organ of the digestive tract consists of these four layers, the layers of different organs show different adaptations to suit the function of that organ. For example one of the functions of the stomach is mechanical digestion so the muscularis of the stomach contains an extra layer of smooth muscle. In contrast to the epithelia, these layers of smooth muscle and connective tissue originate from the mesoderm of the early embryo.

The respiratory system consists of the trachea which branches to produce the numerous smaller airways of the lungs (Wheater *et al.*, 1979). The respiratory system also has an inner epithelium which is surrounded by connective tissue and smooth muscle. Like the digestive system, the epithelium of the respiratory system originates from the endoderm while the smooth muscle and connective tissue originates from the mesoderm of the early embryo.

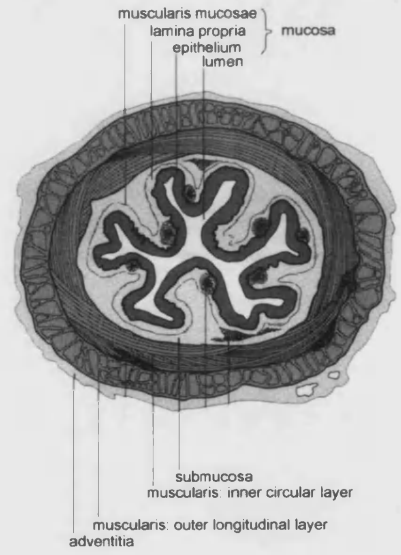
Therefore, the cells of the endoderm give rise to the huge range of cell types that form the epithelia of digestive and respiratory system. In contrast, the layers of connective tissue and smooth muscle which surround these epithelia originate from the mesoderm of the early embryo. This study focuses on the how the endoderm develops to produce the epithelia of the digestive tract and to a lesser extent the epithelia of the respiratory system.

Figure 1.1. Introduction to the early embryo and the gastrointestinal tract. A, Schematic diagram of a transverse section through a stage 14 *Xenopus* embryo. The 3 embryonic germ layers and the archenteron cavity are highlighted. Dorsal is at the top and anterior to the left. B, The gastrointestinal tract. The drawing shows the layered organisation that is common to the organs of the gastrointestinal tract. The drawing in B is adapted from (Wheater *et al.*, 1979).

A


ectoderm
mesoderm
endoderm
archenteron

B


muscularis mucosae
lamina propria
epithelium
lumen
mucosa
submucosa
muscularis: inner circular layer
muscularis: outer longitudinal layer
adventitia

1.3 ENDODERM FORMATION

A first step in the development of the endoderm is that a group of embryonic cells must be instructed to form endoderm rather than one of the other germ layers. This instructive signal could be mediated by at least two possible mechanisms. One possibility is that an intercellular signalling molecule is synthesised by one set of cells and signals to induce endoderm in another set of cells. Alternatively, the signal could be mediated by an intracellular cell fate determinant. The cell fate determinant would be specifically localised to the presumptive endoderm cells and would instruct these cells to form endoderm. Work in *Xenopus* embryos suggests that both these possible mechanisms play a role in endoderm formation.

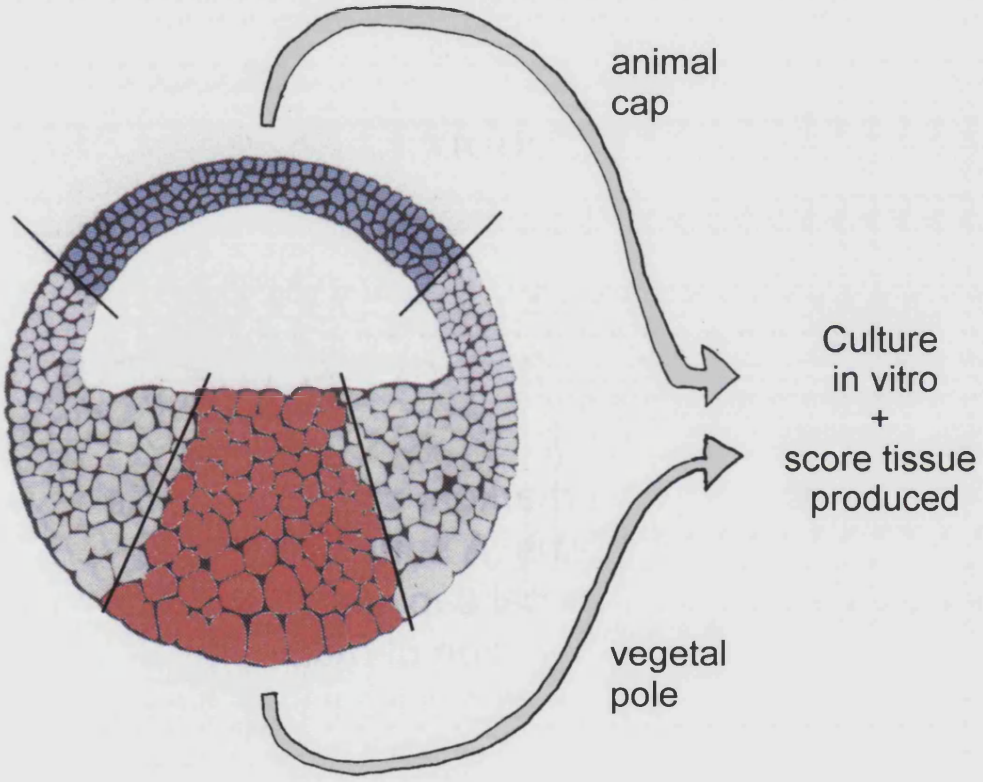
The study of endoderm formation in *Xenopus* was initially hampered because the vegetal pole cells that are fated to form the endoderm in *Xenopus* (Dale and Slack, 1987) do not differentiate well in culture (Holtfreter, 1938a; Holtfreter, 1938b). This made it difficult to score the presence or absence of endoderm in an experiment. The cloning of three molecular markers for the endoderm, *Xlhbox8, IFABP* and *endodermin,* overcame this problem. *Xlhbox8* is a homeodomain containing transcription factor that is expressed in the pancreatic and small intestinal endoderm (Wright *et al.*, 1988). *Xenopus intestinal fatty acid binding protein* (*IFABP*) is expressed in the intestinal endoderm (Shi and Hayes, 1994) and *Endodermin* is a putative protease inhibitor that is expressed throughout the endoderm during early development (Sasai *et al.*, 1996). In addition a monoclonal antibody, 4G6, that is specific to the endoderm has also been used (Jones *et al.*, 1993). These molecular markers made it possible to clearly identify the presence or absence of endoderm in an experiment which has lead to rapid progress in the study of endoderm formation.

Two assays, the animal cap assay and the vegetal pole explant, have been used together with these markers to study endoderm formation (Fig. 1.2). The animal cap assay involves explanting animal cap tissue from blastula stage embryos and growing the animal cap *in vitro*. The type of tissue produced by the animal cap can then be scored by histology or by the expression of molecular markers. Untreated animal

caps will form epidermis but they can be induced to form mesodermal or neural tissues (discussed in Slack, 1991). Animal caps were also found to be capable of being induced to form endoderm by members of the transforming growth factor β (TGFβ) superfamily (Henry *et al.*, 1996; Jones *et al.*, 1993). This means that animal caps can be used as an assay for factors that will induce endoderm. The second assay that has been extensively used in the study of endoderm development is the vegetal pole explant (Fig. 1.2). This assay is similar to the animal cap assay but explants are cut from the vegetal pole and cultured *in vitro*. Although these explants undergo poor histological differentiation they were found to autonomously express molecular endodermal markers (Gamer and Wright, 1995; Henry *et al.*, 1996; Jones *et al.*, 1993). In *Xenopus* it is not possible to use genetic approaches to knockout the function of a gene. However, it has been possible to block the activity of some signalling molecules by using dominant negative receptors (Amaya *et al.*, 1991). Ectopic expression of a dominant negative activin receptor in vegetal pole explants blocked the expression of *Xlhbox8* (Henry *et al.*, 1996). This showed that vegetal pole explants can be used to assay for factors that inhibit or alter endoderm formation.

The fact that members of the TGFβ superfamily can induce endoderm in animal caps suggests that they may play a role in induction of the endoderm *in vivo*. The expression of the dominant negative activin receptor also inhibited the expression of endodermal markers in vegetal pole explants. This provided further evidence that a member of the TGFβ superfamily is important for endoderm formation *in vivo*. However, the dominant negative construct is not specific to a particular TGFβ superfamily member so it was not possible to say which TGFβ was responsible. The debate over possible roles of different members of the TGFβ superfamily in endoderm induction has continued.

Figure 1.2. The animal cap and vegetal pole assay. The animal cap assay involves cutting out the animal cap and growing it *in vitro*. The type of tissue produced by the animal cap is then scored. The vegetal pole assay is very similar but involves cutting out the vegetal pole of the embryo instead of the animal cap. The vegetal pole is then grown *in vitro* and the type of tissue produced is scored. Part of this figure has been adapted from Wolpert, 1998.


animal
cap
Culture
in vitro
+
score tissue
produced
vegetal
pole

A combination of these two assays and experiments on whole embryos has been used to study a number of transcription factors that have been found to be important in the early development of the endoderm. *Xsox17α* and *Xsox17β*, two members of the sox family of high motility group (HMG) box containing transcription factors, have been shown to be expressed in the endoderm (Hudson *et al.*, 1997). They can induce the expression of *endodermin* and *Xlhbox8* in animal caps and are induced by activin a member of the TGFβ superfamily which is capable of inducing endoderm. In order to study the loss of function of a transcription factor in *Xenopus* it is possible to produce an antimorphic form of the transcription factor. If the transcription factor is an activator of transcription then a hybrid molecule is produced that has the DNA binding domain of the transcription factor fused to the repressor domain from the transcription factor *engrailed* (Conlon *et al.*, 1996). Alternatively, if the transcription factor is normally a repressor of transcription then the DNA binding domain is fused to the VP16 activation domain. Blocking the function of *Xsox17β*, using this approach, inhibits the activin induced expression of *endodermin* and *Xlhbox8* in animal caps. Therefore, *Xsox17α* and *Xsox17β* seem to play an important role in the formation of the endoderm.

Members of the paired family of homeodomain containing transcription factors have also been shown to be involved in endoderm formation. *Mix1* is expressed in the vegetal hemisphere once zygotic transcription starts at mid blastula transition (Rosa, 1989). *Mix1* is activated by activin and when overexpressed is capable of inducing *endodermin* in animal caps (Lemaire *et al.*, 1998). Inhibiting the function of *Mix1*, by overexpression of a *Mix1* engrailed repressor fusion construct reduced *endodermin* expression in whole embryos. Two other members of the paired family of homeodomain containing transcription factors also play a role in endoderm formation. *Milk* (Mix Like) is expressed in the presumptive endoderm and has been shown to be induced by activin (Ecochard *et al.*, 1998). Overexpression of *Milk* induces *endodermin* and inhibits mesodermal markers in whole embryos. *Mixer*, a third member of the paired family of homeodomain containing transcription factors, is expressed in the endoderm during gastrulation and can induce *endodermin*, *IFABP*, *Xlhbox8* and *Xsox17α+β* in animal caps (Henry and Melton, 1998). *Mixer* can be

induced by *Vg1*, a member of the TGFβ superfamily discussed below, and the engrailed repressor form of Mixer blocks the *Vg1* induced activation of endodermal markers in animal caps. Therefore, *Xsox17α*, *Xsox17β*, *Mix1*, *Milk* and *Mixer* are all good candidates for mediating the signal responsible for endoderm formation.

The expression of these transcription factors all start soon after the onset of zygotic transcription at mid blastula transition. It seems likely that they are activated by a maternal signal and that they then activate a cascade of genes required for endoderm development. At present there are two maternally expressed genes that are good candidates for providing this activation signal. The first candidate is *Vg1*, which is a member of the TGFβ superfamily that is capable of inducing endodermal markers. *Vg1* mRNA is maternally expressed and localised to the vegetal hemisphere in *Xenopus* eggs (Weeks and Melton, 1987). Recently mutant forms of the ligand have been developed that have a specific dominant negative effect on *Vg1* signalling (Joseph and Melton, 1998). Expression of these ligands blocks the expression of *Xlhbox8* showing that *Vg1* activity is required for correct endoderm development. These factors make *Vg1* a good candidate for a maternal signal that is responsible for endoderm formation.

A second candidate for a maternal factor that activates endodermal markers is the transcription factor *VegT* (Zhang and King, 1996), which has also been called *Antipodean* (Stennard *et al.*, 1996), *Xombi* (Lustig *et al.*, 1996) and *Brat* (Horb and Thomsen, 1997). The maternally expressed *VegT* mRNA is localised to the vegetal pole of the egg and overexpression of *VegT* is capable of inducing endodermal markers in animal caps (Horb and Thomsen, 1997). Antisense ribonucleotides were used to deplete the maternally expressed *VegT* mRNA (Zhang *et al.*, 1998). This reduction of maternal *VegT* mRNA caused a reduction in the expression of *Xsox17α*, *Xlhbox8*, *endodermin* and *IFABP*. This suggests that maternal *VegT* has a key role as a cell fate determinant, that activates the expression of the transcription factors expressed in the endoderm, thus causing endoderm formation.

Several signalling molecules expressed in the organiser region have also been shown to have an affect on endoderm formation. *Noggin* and *chordin* are both secreted molecules that are expressed in the organiser region (Sasai *et al.*, 1994; Smith and Harland, 1992). Ectopic expression of either gene causes the induction of *Xlhbox8* and *endodermin* in animal caps (Sasai *et al.*, 1996). *Cerberus* is expressed in the anterior organiser and when ectopically expressed is capable of duplicating anterior structures such as the head and the liver (Bouwmeester *et al.*, 1996). *Cerberus* overexpression can also induce *endodermin* expression in animal caps. The ability of these 3 molecules, that are secreted by the organiser, to induce endoderm suggests that they have a role in endoderm development *in vivo*. However, what that role is remains to be established.

Recent work has further clarified the hierarchy of events that occurs during the formation of the endoderm. Cell disassociation/reassociation experiments were used to establish whether the expression of these endodermal markers requires cell contact (Yasuo and Lemaire, 1999). Endoderm induction was found to occur in two phases. The first phase results in the activation of *Xsox17α* and *Mix1* at mid blastula transition. This phase does not require cell-cell contact or protein synthesis. This cell autonomous activation is most likely caused, at least in part, by the maternally expressed *VegT*. The second phase in endoderm induction causes the activation of *Mixer* and the further accumulation of *Xsox17α* and *Mix1* expression. This phase requires cell contact and protein synthesis, so it is likely to involve a signalling molecule. The *nodal* related factors, *Xnr-1* and *Xnr-2* (Jones *et al.*, 1995), are also members of the TGFβ superfamily. They are induced by *VegT*, expressed at the right time and place and can induce the expression of *Mixer*, *Xsox17α* and *Mix1*. This makes the *nodal* related factors the best candidates for mediating the second phase of endoderm formation.

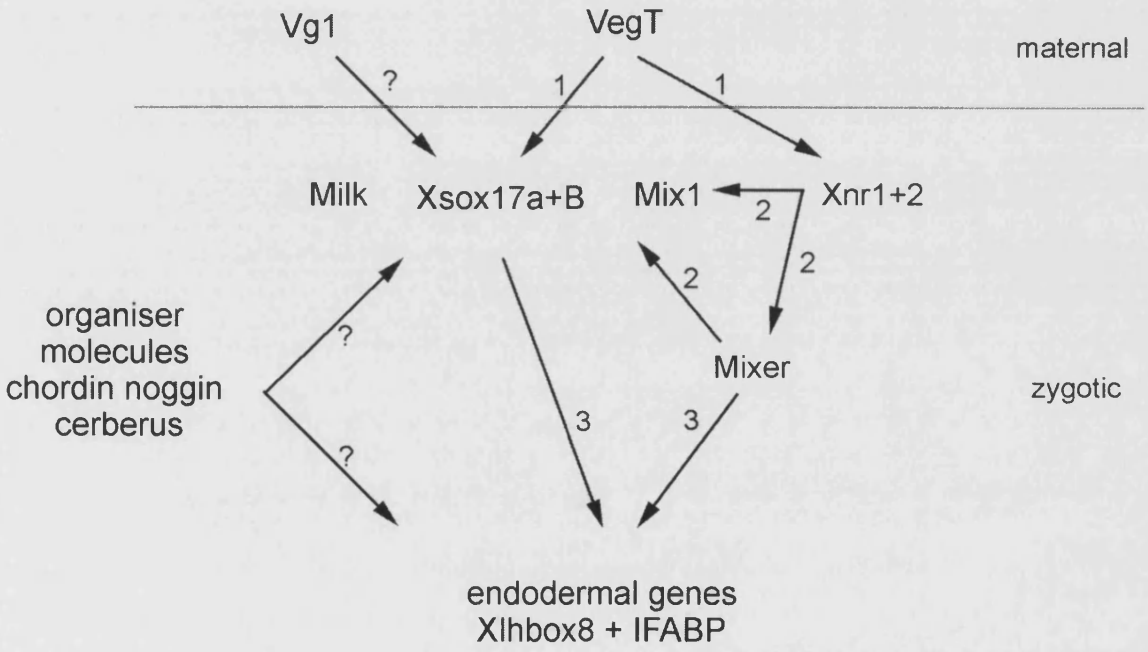
A model for the formation of the endoderm. The results from these studies can be combined to produce a model that is one interpretation of the current understanding of endoderm induction (Fig. 1.3). Maternally expressed *VegT* and *Vg1* mRNA is localised to the vegetal pole of the egg and early embryo. At mid blastula transition

VegT activates the expression of *Xsox17α+β, Mix1, Xnr1, Xnr2* and possibly *Milk.* This is the first step in the formation of the endoderm. *Vg-1* may also play a role in activating these genes. The second step of endoderm formation occurs during later blastula stages when the nodal related TGFβs activate *Mixer* and cause the further accumulation of *Xsox17α+β* and *Mix1*. At gastrula stages signals from the organiser may cause further up regulation of these genes or possibly play a role in patterning the endoderm (discussed below). These transcription factors then activate a cascade of genes, e.g. *Xlhbox8*, *endodermin* and *IFABP*, that are involved in the development and differentiation of the endoderm. This is labelled step 3 but is very likely to involve multiple steps.

This model summarises the current ideas regarding endoderm induction but it is important to understand that this is a very active field. Future work is likely to identify many other molecules that are involved in induction of the endoderm. For example, it has just been shown that *Bix4,* another member of the paired family of homeodomain containing transcription factors, is also a target of maternal *VegT* and plays an important role in the formation of the endoderm (Casey *et al.*, 1999).

Further work will also be required to confirm the details and overcome the limitations of current work. For example a limitation of a lot of these experiments is that their results are based on a very small number of endodermal markers. The problems that this can cause are clear when *Vg1* is considered as an example. The dominant negative *Vg1* ligand blocks the expression of *Xlhbox8* in vegetal pole explants. This was interpreted as showing that *Vg-1* blocks endoderm formation. However, a lack of *Xlhbox8* expression does not show a lack of endoderm and it could be that the dominant negative ligand changes the type of endoderm that is present to a non *Xlhbox8* expressing endoderm. This means that *Vg1* may play a role in patterning rather than induction of the endoderm. Many of the experiments discussed here suffer from this limitation and it seems likely that as more region specific endodermal markers are characterised some of the molecules discussed here

Figure 1.3. Formation of the endoderm. A schematic diagram shows the molecules currently thought to be responsible for the formation of the endoderm. See text for details.


Vg1
VegT
maternal
?
1
1
Milk
Xsox17a+B
Mix1
Xnr1+2
2
2
2
organiser
molecules
chordin noggin
cerberus
?
?
Mixer
zygotic
3
3
endodermal genes
Xlhbox8 + IFABP

will be found to play a role in the regional specification, rather than the induction of the endoderm. The secreted factors *chordin* and *noggin* are good examples of factors that may play a role in patterning the endoderm.

Another issue that remains to be addressed is how endoderm induction in *Xenopus* relates to endoderm formation in other organisms. There has not been as much work carried out on the formation of the endoderm in other organisms. However, in Zebrafish embryos as in *Xenopus* TGFβ signalling has been shown to be required for correct endoderm formation (Peyrieras *et al.*, 1998). This demonstrates that at least some of the mechanisms of endoderm formation have been conserved during evolution. Once more studies have been carried out in different organisms, comparisons of the mechanisms in different species will provide valuable insights into the mechanisms of endoderm formation.

1.4 REGIONAL SPECIFICATION OF THE ENDODERM 1: AMPHIBIAN RECOMBINATION EXPERIMENTS

Another key process in the development of the endoderm is that of regional specification. Regional specification is the division of the endoderm, by instructive signals, into sub populations of cells that are fated to form each individual organ. The mesoderm has been found to play an important role in the regional specification of the endoderm in a number of experimental systems.

The role of the mesoderm in patterning the endoderm was addressed in classical experiments using tissue from the newt, *Triturus pyrrhogaster*. Pieces of early endoderm and mesoderm were dissected from blastula and neurula embryos and grown *in vitro*, either separately or recombined together (Fig. 1.4A). The tissue produced by the explants was then established by looking at the histology of the explants. These recombination experiments demonstrated that explanted endoderm will only develop to produce differentiated endodermal organs when it is cultured in the presence of mesoderm (Fig. 1.4A) (Okada, 1954a; Okada, 1954b; Okada, 1955a; Okada, 1955b). This shows that the endoderm requires a permissive signal from the

mesoderm for its development. A permissive induction is one that allows a cell to differentiate according to its fate but does not instruct the cell as to what that fate should be (permissive and instructive inductions are discussed in Slack, 1991). For example, a piece of endoderm could be fated to form stomach but might remain undifferentiated endoderm if it does not receive signal X. Signal X is then a permissive induction because it "permits" the endoderm to differentiate but does not tell the endoderm what its fate should be.

The type of mesoderm was then shown to affect the type of endoderm produced in the recombination experiments (Fig. 1.4A) (Okada, 1955a; Okada, 1955b; Okada, 1957; Okada, 1960). Endoderm that was fated to form pharynx would produce pharynx when combined and cultured with dorsal or anterior mesoderm. However, the same endoderm combined with lateral mesoderm was found to produce posterior organs such as intestine. This suggested that mesodermal signals are not only capable of permissive induction but are capable of changing the fate of the endoderm, an instructive induction. Signals from different regions of the mesoderm may therefore pattern the endoderm so that different regions of the endoderm produce different organs. A second important finding of this work was that the origin of the endoderm was important in deciding what endodermal organs were produced. For example, posterior endoderm was more likely to produce intestine than anterior endoderm. This shows that, although the mesoderm is important in patterning the endoderm, the neurula endoderm already has some regional organisation before the action of the mesoderm.

When considering these classical experiments it is worth remembering that they suffer from a number of important limitations. At the time of these early recombination experiments it was impossible to label one of the recombined tissue explants. This meant that there was no way of proving that a particular tissue came from explanted endoderm and not from endoderm contaminating the explanted mesodermal tissue. This is a severe limitation to these early experiments. A second limitation to these early recombination experiments is that they were carried out without an accurate fate map. If the fate of the explant in normal development is not

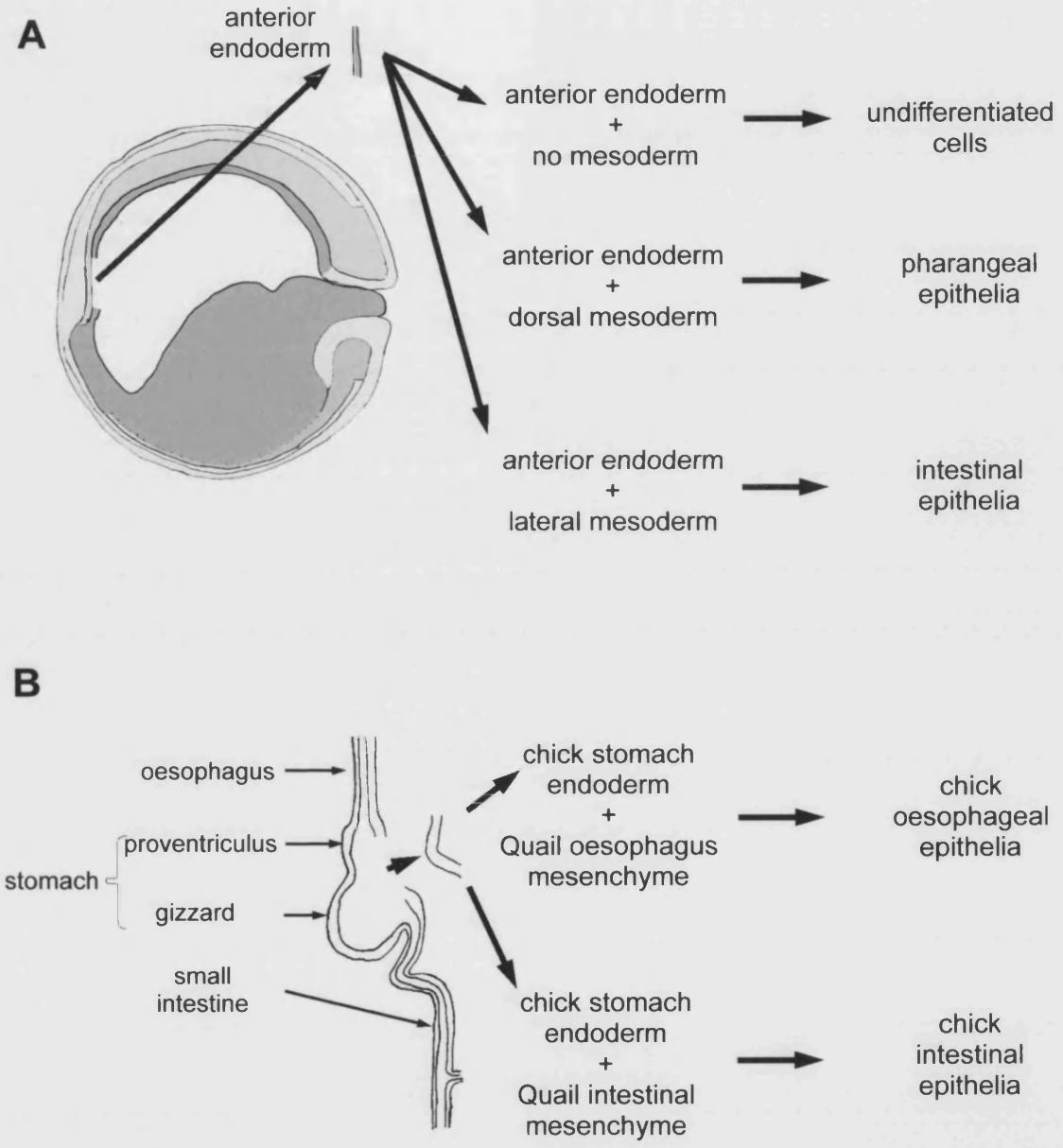
known there is no way of knowing if the fate of the endoderm has been changed by the mesoderm. Therefore, an accurate fate map is essential for interpreting the results of this kind of experiment. Thirdly, of course, at the time of these experiments it was not possible to analyse these inductions at the molecular level.

1.5 REGIONAL SPECIFICATION OF THE ENDODERM 2. THE CHICK/QUAIL RECOMBINATION EXPERIMENTS

The mesoderm has also been shown to be able to respecify the fate of the endoderm in chick embryos (Fig. 1.4B). Explants of 5 day old chick endoderm were recombined with 5 day old quail mesoderm from different digestive organs (Yasugi and Mizuno, 1978). The recombinations were cultured on the extraembryonic chorio/allantoic membrane of another embryo and the differentiation of digestive organs scored based on their histology. The stomach endoderm was found to be able to be respecified to either oesophagus or intestine by the appropriate heterologous mesenchyme (Fig. 1.4B). Later work showed that the stomach explants that had been converted to an intestinal fate expressed the intestinal enzyme sucrase (Ishizuya-Oka and Mizuno, 1984) and a range of endocrine hormones normally expressed by the intestine (Andrew and Rawdon, 1990). This confirmed at the molecular level that the transformation was occurring. In the amphibian experiments the origin of the endoderm had an effect on the type of epithelia produced. The origin of the endoderm was also found to be important in the chick because the small intestine and oesophageal endoderm could not be respecified by heterologous mesenchyme.

A range of other tissues were shown to be capable of being respecified or causing respecification in chick embryos. The chick stomach is divided into two distinct regions (Fig. 1.4B). The proventriculus has compound glands and secretes digestive enzymes while the gizzard has simple tubular glands and a mechanical function (Takiguchi *et al.*, 1986). Gizzard endoderm when combined with proventricular mesoderm was found to develop into a proventricular epithelium (Takiguchi *et al.*, 1986). The transformation to a proventricular fate was confirmed at the molecular

Figure 1.4. Mesoderm/endoderm recombination experiments. A, amphibian recombination experiments. Early endoderm is explanted from the embryo and recombined with mesoderm from the early embryo. The recombination is cultured *in vitro* and then the type of epithelia produced by the endoderm is scored. B, chick/quail recombination experiments. Stomach endoderm is cut from the developing chick gut and recombined with quail gut mesenchyme. The recombination is grown on an extraembryonic membrane and the type of epithelia produced by the endoderm is scored.


A
anterior endoderm
anterior endoderm + no mesoderm
undifferentiated cells
anterior endoderm + dorsal mesoderm
pharangeal epithelia
anterior endoderm + lateral mesoderm
intestinal epithelia
B
oesophagus
proventriculus
stomach
gizzard
small intestine
chick stomach endoderm + Quail oesophagus mesenchyme
chick oesophageal epithelia
chick stomach endoderm + Quail intestinal mesenchyme
chick intestinal epithelia

level as the explants expressed the proventricular marker, embryonic chick pepsinogen (Takiguchi *et al.*, 1986) and proventricular endocrine hormones (Rawdon and Andrew, 1988). The opposite transformation can also occur as proventricular endoderm could be respecified to a gizzard fate by gizzard mesoderm.

Mesoderm was also found to be able to have an inductive effect *in vivo* on endoderm that is fated to be extraembryonic. Quail allantoic endoderm, which is normally fated to form an extraembryonic membrane, was implanted into the presumptive digestive area of host chick embryos. The grafts were located in later development and their differentiation scored based on their histology and the expression of tissue specific antigens (Yasugi, 1984). The allantoic endoderm was found to develop into appropriate differentiated epithelia according to its position in the mesoderm, showing that the mesoderm could respecify the fate of the extraembryonic allantois.

One of the limitations of the amphibian work was that it could not be proved that the epithelia was formed from the explanted endoderm rather than from contaminating endoderm in the mesoderm explants. The cells from the chick and quail can be easily distinguished from each other (Le Douarin, 1969) so the chick/quail recombination experiments do not suffer from this limitation. A second limitation of the amphibian experiments was the lack of an accurate fate map. In the chick experiments the problem of prospective fate was overcome by taking explants once the individual digestive organs could be distinguished based on their anatomy. This highlights two fundamental differences between the amphibian and chick experiments (Fig. 1.4A+B). The first difference is that because the chick experiments require this anatomy based identification they are limited to studying endoderm development at much later stages than the amphibian experiments. The second difference is that the amphibian experiments recombined endoderm with early mesoderm, which would have a range of fates such as head mesenchyme, notochord or smooth muscle. In contrast the chick experiments combine the endoderm with mesenchyme that has separated from the rest of the mesoderm and is fated to only form smooth muscle and connective tissue. It is worth remembering that, although both sets of experiments

show that the mesoderm is capable of causing the respecification of the endoderm, there are these important distinctions between these two types of experiments.

The chick mesenchyme is clearly able to cause the respecification of the endoderm to a new fate based on histology and the expression of molecular markers. The question of whether the mammalian mesenchyme has the same properties is not so well established. Explant experiments with stomach endoderm from mouse embryos showed that the endoderm required a permissive induction from the mesenchyme for its differentiation (Fukamachi *et al.*, 1979). However the heterologous mesenchyme did not cause the respecification of the endoderm which differentiated according to its presumptive fate. Rat endoderm was also found to be resistant to respecification by heterologous mesenchyme (Dulac *et al.*, 1994; Fukamachi and Takayama, 1980). An exception was the rat colonic endoderm which was found to be respecified to a small intestinal fate by small intestinal mesenchyme (Dulac *et al.*, 1994). It has also been shown that rat intestinal fibroblasts can cause the respecification of chick gizzard endoderm to an intestinal fate (Haffen *et al.*, 1983). This suggests that mammalian mesenchyme, like the chick mesenchyme, has the ability to cause the respecification of the endoderm but at the time point that these experiments were carried out the endoderm had lost its ability to respond. The size of the early mammalian embryo has meant that this hypothesis has not been tested by carrying out experiments with earlier endoderm. In support of this hypothesis, it is known that as the chick endoderm gets older it loses its ability to respond to heterologous mesenchyme (e.g. Ishizuya-Oka and Mizuno, 1984; Matsushita, 1996a; Takiguchi *et al.*, 1988). An alternative hypothesis is that the lack of response is caused by differences in the mesenchyme, and presumably developmental mechanism, between chick and mammalian embryos.

The ability of the chick mesenchyme to cause the respecification of explanted endoderm has clearly been well established but it is only recently that there has been any progress in understanding the molecular mechanisms responsible for this induction. The *Hox* genes are a large family of homeobox containing transcription factors that are involved in the anterior/posterior patterning of the ectoderm and

mesoderm (reviewed by Krumlauf, 1994). Most vertebrates have four clusters of *Hox* genes, termed *Hoxa* to *Hoxd.* Each cluster contains most but not all of the members of 13 paralogue groups. In the axial tissues, each member of the *Hox* gene family is expressed from the posterior of the embryo up to a different anterior position. This produces a nested expression pattern emerging from the posterior of the embryo.

In the 3 day old chick gut before the formation of any anatomical boundaries a number of *Hox* genes were found to be expressed in a nested pattern in the gut mesenchyme (Roberts *et al.*, 1995; Yokouchi *et al.*, 1995). The genes were members of the posterior paralogue groups 9 to 13. At later stages of development when the anatomically defined organs of the gut have formed the expression pattern of the *Hox* genes had changed. The expression patterns were no longer nested but different *Hox* genes were expressed in partially overlapping domains. For example, *Hoxa9* was expressed in the mesenchyme of the small intestine and the cecum (the cecum lies between the small and large intestine and is analogous to the mammalian appendix), *Hoxa10* in the mesenchyme of the cecum, *Hoxa11* in the mesenchyme of the large intestine and the cloaca and *Hoxa13* was only expressed in the cloaca mesenchyme. Therefore, the boundaries in *Hox* gene expression in the mesenchyme was found to match anatomical boundaries in the epithelia. This suggested that the boundaries of *Hox* gene expression in the mesenchyme initiate a signal that patterns the underlying endoderm. This idea was partially confirmed by using viral mediated ectopic expression of *Hoxd13* in the presumptive intestinal mesoderm (Roberts *et al.*, 1998). Ectopic expression of *Hoxd13* was found to cause a transformation of the small intestinal epithelia into a large intestinal epithelia. Interestingly the epithelia still expressed markers for small intestine such as sucrase so the transformation was only partial. This is the first example in the vertebrate gut of a molecule expressed in the mesenchyme that is capable of causing a transformation in the underlying endoderm.

The regional expression of the Hox genes in the chick mesenchyme bares a striking resemblance to the situation in the midgut of *Drosophila melanogaster.* In the mesenchyme of the *Drosophila* midgut three Drosophila *Hox* genes, *Antennapedia (Antp), Ultrabithorax (Ubx)* and *abdominal-A* (*abd-A*) are expressed in adjacent

domains (Bienz, 1994). These *Hox* genes activate the expression of the signalling molecules *decapentaplegic (dpp),* a member of the TGFβ superfamily, and *wingless (wg)*, a member of the *wnt* family, in the gut mesenchyme. Dpp and wg then activate the expression of another *Hox* gene, *labial (lab)* (Bienz, 1994), and a member of the AP-1 family of transcription factors, *Dfos* (Riese *et al.*, 1997) in the underlying mid gut endoderm. Labial then goes on to cause the specification of a single intestinal cell type, the copper cells (Hoppler and Bienz, 1994). Therefore, in *Drosophila* as in the chick, there are domains of *Hox* gene expression in the mesenchyme that play a role in patterning the underlying endoderm. In *Drosophila* it is known that the *Hox* genes activate the expression of *dpp* and *wg* which signal to the endoderm to activate *Dfos* and *labial.* In contrast, in chick embryos the molecules responsible for the signal from the mesoderm and the responding signals in the endoderm remain unknown. It is tempting to speculate that homologues of the molecules involved in *Drosophila* may also be involved in the chick.

Although the mesenchyme of the chick is capable of causing the respecification of the endoderm and some progress is being made in explaining the molecular mechanism behind this respecification, it is still not clear what role the proposed signals play in normal development. A simple model is that during development the mesoderm becomes patterned, and once the gut tube has formed, the gut mesenchyme is responsible for patterning the previously unpatterned endoderm. A problem with this model is that both the chick and amphibian experiments suggest that the endoderm has at least some regional specification before the action of the mesoderm (further experiments discussed below also suggest this). If the endoderm is already patterned then what is the role of the signals from the mesenchyme? This problem is well illustrated by the expression of *CdxA* in chick embryos (Ishii *et al.*, 1997). *CdxA* is a homeobox containing transcription factor that is expressed in the intestinal epithelium. Intestinal mesenchyme can respecify stomach endoderm to an intestinal fate, so not surprisingly, intestinal mesenchyme can induce *CdxA* expression in stomach endoderm that would not express it during normal development. The problem lies in the fact that in normal development the expression of *CdxA* starts in the presumptive intestinal endoderm before the gut tube forms and

becomes covered in mesenchyme. This means that in normal development the initial expression of C*dxA* can not be induced by the intestinal mesenchyme. These results raise at least two questions. First, what is the role of these signals from the mesenchyme if the endoderm already contains pattern. Second, what is responsible for the early patterning of the endoderm.

It is possible to come up with at least two possible explanations to these questions. One possibility is that the cells that will form the mesenchyme could signal to the endoderm and induce pattern early in development, before they separate from the rest of the mesoderm. These presumptive mesenchyme cells would then continue to signal to the endoderm providing further pattern as they separate from the rest of the mesoderm and form the gut mesenchyme. In this model the cells that form the mesenchyme provide continuous signals to the endoderm. If the future gut mesenchyme is not providing early signals, then the endoderm could contain a crude amount of patterning from an alternative and unknown mechanism. This early pattern could then be refined by later signals from the mesenchyme to establish sharp boundaries between organs.

In summary, the chick/quail recombination experiments have shown that the gut mesenchyme can cause the respecification of the endoderm. However, the molecular mechanisms responsible for this respecification and the role of these signals *in vivo* remains to be established.

1.6 REGIONAL SPECIFICATION OF THE ENDODERM 3: INDUCTION OF THE PANCREAS AND LIVER

In the amphibian recombination experiments the early mesoderm causes the respecification of the endoderm while in the chick the mesenchyme that surrounds the intestinal tract causes the respecification of the endoderm. Two other mesodermal tissues, the notochord and cardiac mesoderm, have also been shown to provide inductive signals that are important in the development of the endoderm.

The pancreas develops from a dorsal and a ventral bud which segregate from the rest of the endoderm, fuse and differentiate to give rise to large number of exocrine and endocrine cell types (reviewed in Slack, 1995). Explant experiments have shown that like the other organs of the digestive tract, the pancreas requires the presence of its mesenchyme for differentiation to occur (Wessells and Cohen, 1967). However, it has also been shown that development of the dorsal pancreatic bud requires a signal from the notochord (Hebrok *et al.*, 1998; Kim *et al.*, 1997). This signal acts by inhibiting the expression of *sonic hedgehog* in the pancreatic endoderm which allows pancreatic development to continue. This signal is a permissive induction because although it is required for pancreas development, notochord is not capable of inducing pancreas development in other regions of the endoderm.

The liver endoderm also requires its mesenchyme, the hepatic mesenchyme, for the differentiation of hepatocytes, the major cell type of liver epithelium (Le Douarin, 1975). Interestingly, an earlier signal which comes from the cardiac mesoderm is also required for the induction of the liver (Le Douarin, 1975). It has recently been shown that members of the fibroblast growth factor family (FGF) expressed by the cardiac mesoderm provide the signal that is responsible for liver induction (Jung *et al.*, 1999).

These experiments show that the development of the pancreas and liver require early signals from specific parts of the mesoderm, notochord or cardiac mesoderm, as well as later signals from the gut mesenchyme. It seems likely that other endodermal organs may also need sequential signals from different mesodermal tissues as they develop form early endoderm to differentiated epithelia

1.7 REGIONAL SPECIFICATION OF THE ENDODERM 4: PATTERNING WITHOUT THE MESODERM

A common theme of the chick and amphibian recombination experiments is that, although the mesoderm has the ability to cause the respecification of the endoderm, different parts of the endoderm have different responses to the signal. This shows

that the endoderm has at least some prepattern before it receives signals from the mesoderm. Data from a number of other experiments also show that the endoderm contains pattern before the action of the mesoderm.

Vegetal pole explants (Fig. 1.2) from *Xenopus* embryos autonomously express the antigen for an endoderm specific antibody, *Xlhbox8*, *IFABP* and *cerberus* (Gamer and Wright, 1995; Henry *et al.*, 1996; Jones *et al.*, 1993; Zorn *et al.*, 1999). However, *Xlhbox8* and *cerberus* are only expressed in the dorsal part of the vegetal pole explants and the ectopic expression of the dominant negative *activin* receptor was found to block *Xlhbox8* but not *IFABP* expression. Both these observations show that there are regional differences in the endoderm of the vegetal pole explants suggesting that before gastrulation the *Xenopus* endoderm has at least some pattern or the ability to produce it. A crucial assumption of these experiments is that the vegetal pole explants contain no contaminating mesoderm. A bad dissection could take tissue from the marginal zone which may then form mesoderm and affect the development of the endoderm. Some of the studies showed that contaminating mesoderm was not present by checking for the expression of markers for the dorsal, lateral and ventral mesoderm (Henry *et al.*, 1996) but not all the studies were this rigorous. The potential problem is exacerbated because the results are often scored by using RT-PCR (reverse transcriptase-polymerase chain reaction) on pools of vegetal pole explants. If one in ten explants expresses endodermal markers because it contains contaminating mesoderm RT-PCR would be sensitive enough to give a positive result. Aside from this potential problem, the vegetal pole explants strongly suggest that prior to gastrulation the endoderm has some regionalisation or at least the autonomous ability to produce it.

Another example of pattern in the endoderm comes from embryos of the aquatic salamander, the axolotol *Ambystoma mexicanum* (Barlow and Northcutt, 1997). Explants of anterior endoderm were taken from neurula stage embryos and grown *in vitro* without mesoderm. The explants were found to autonomously develop taste buds showing that this specialised endodermal cell type does not need mesoderm to differentiate in the correct region of the endoderm.

There is also evidence that the endoderm of the chick embryo is patterned and under certain conditions can undergo differentiation in the absence of mesoderm (Sumiya, 1976a; Sumiya, 1976b). Explants of endoderm were taken from 1-2 day old chick embryos. The explants were then wrapped in a fragment of vitelline membrane and cultured *in vitro* with serum added to the culture medium. (Ishizuya-Oka, 1983; Sumiya, 1976b). The endoderm fragments were found to produce differentiated epithelia without mesoderm. The production of differentiated epithelia was later confirmed using electron microscopy and the expression of alkaline phosphatase as a marker of intestinal epithelia (Ishizuya-Oka, 1983).

Just after gastrulation (stage 4), epithelia of the pharynx, oesophagus, stomach and small intestine were identified in the explants. This is much earlier than the explants of endoderm that were taken for the recombination experiments and shows that these organs can be specified and differentiate without the influence of mesenchyme of the digestive tract. At this early stage, the chick embryo is too small to split the endoderm into more than two regions. This means that it is not possible to show whether the endoderm has the ability to produce pattern during culture or if it is already patterned at the time the explants were taken.

Explants taken from later stages, although still earlier than used for the recombination experiments, contained differentiated epithelia of the pancreas, liver and large intestine as well as the organs found in the earlier explants. This shows that as the endoderm ages, progressively more organs become specified. At these later stages it was possible to split the endoderm into more regions. The organs produced by an explant matched the position the explant was taken from, so pharynx would develop in anterior explants and intestine would develop in posterior explants. Therefore the chick endoderm seems to show increasing regional specification before the stages used in the mesenchyme respecification experiments.

The big unanswered question regarding these experiments is the role of the vitelline membrane and the serum. They are both required for the endoderm to differentiate

and so seem to provide the permissive induction that is normally provided by the mesoderm. This permissive induction allows the endoderm to develop according to its presumptive fate but how the vitelline membrane and serum provide this signal is unknown.

In summary, the chick and amphibian endoderm both seem to have some regional specification before they receive signals from the mesoderm but how this patterning is brought about and how it fits in with the signals from the mesoderm is not known.

1.8 REGIONAL SPECIFICATION OF THE ENDODERM 5: SUMMARY

The chick recombination experiments show that the gut mesenchyme has the ability to cause the respecification of the endoderm and there has been some progress in explaining the molecular mechanisms responsible for this transformation. However, the actual function of these signals *in vivo* remains to be established. There is also evidence that the endoderm contains at least some pattern before the action of the gut mesenchyme but it is not known what factors are responsible for this early patterning of the endoderm. It is also not known how the early patterning relates to the later signals from the mesenchyme. Therefore, there are numerous aspects of the regional specification of the endoderm that are not understood.

The amphibian experiments also showed that the mesoderm has the ability to cause the respecification of the endoderm. The advantages of the amphibian system (see chapter 1.11) make it well suited for trying to establish the mechanisms responsible for the regional specification of the endoderm. However, the classical amphibian experiments suffered from a number of important limitations. At the time of these experiments it was not possible to show that the epithelia produced by the recombinations did not come from contaminating endoderm. Modern cell lineage labels mean that this can now be carried out (Gimlich and Braun, 1985). A second limitation of these experiments was that they could not make use of modern molecular technology. It would now be possible to use molecular markers to show the presence of endoderm or to investigate the role of candidate genes in the patterning of the endoderm. The final limitation of these classical experiments was

that they were carried out without an accurate fate map. A fate map is required so that the result of any explant or transplantation experiment can be compared to the presumptive fate for the explanted piece of tissue. Currently, *Xenopus* is the most widely used amphibian for the study of developmental biology and prior to this study there was no fate map for the *Xenopus* endoderm.

1.9 INDUCTIVE SIGNALS FROM THE ENDODERM

The focus so far, has been on signals received by the endoderm but the endoderm also provides inductive signals that pattern tissues from other germ layers.

The most famous signal from the endoderm (strictly, the cells that will form the endoderm) is the mesoderm inducing signal that is secreted by the vegetal pole of amphibian embryos (Nieuwkoop, 1969). This signal induces mesoderm in the overlying marginal zone and was previously thought to be mediated by a maternally expressed intercellular signalling molecule (reviewed in Kimelman and Griffin, 1998). However, reduction of the maternal expression of the transcription factor *VegT,* which blocks the formation of the endoderm (discussed in chapter 1.3), also blocks the mesoderm inducing signal (Zhang *et al.*, 1998). This shows that the mesoderm inducing factor is probably activated by *VegT* at mid blastula transition and that the production of the mesoderm inducing signal is linked to the development of the endoderm.

Another signal from the endoderm is required for heart formation. In *Xenopus* embryos, the anterior dorsal endoderm was shown to induce hearts in explanted mesoderm (Nascone and Mercola, 1995) and removal of this tissue was found to block heart formation in explants and whole embryos. Therefore, a signal from the anterior endoderm is required for heart formation in *Xenopus*. This signal seems to be conserved during evolution as the anterior endoderm has been shown to induce cardiac mesoderm in chick embryos (Orts-Llorca and Gil, 1965).

This anterior dorsal endoderm in *Xenopus* is also thought to play a role in inducing head structures (Bouwmeester *et al.*, 1996). As *cerberus* is expressed in the anterior

dorsal endoderm and misexpression of *cerberus* is capable of inducing ectopic heads, it has been proposed that this region plays a role in head induction. In mice it has been shown that the primitive endoderm is responsible for the patterning of anterior structures (Thomas and Beddington, 1996). It is important to stress that the primitive endoderm is not part of the embryonic endoderm but is an extraembryonic tissue. This primitive endoderm has also been shown to express a transcription factor *hex* and a *cerberus* related molecule (Belo *et al.*, 1997; Thomas *et al.*, 1998). The anterior dorsal endoderm in *Xenopus* expresses *Xhex* as well as *cerberus* (Jones *et al.*, 1999; Zorn *et al.*, 1999). This has led to the hypothesis that the anterior dorsal endoderm in *Xenopus* is the functional equivalent of the primitive endoderm in mice and so, is responsible for head induction in *Xenopus.* However, this remains contentious as recent evidence shows that removal of this anterior dorsal endoderm does not affect head induction (Schneider and Mercola, 1999).

Earlier in this introduction, the ability of the gut mesenchyme to signal to the underlying gut epithelia was discussed. The last example of signals from the endoderm shows that the gut epithelia can also signal to the gut mesenchyme. In rat embryos, explanted epithelium is capable of inducing smooth muscle actin in fibroblasts. (Kedinger *et al.*, 1990) and in explants of tissue from mouse embryos the stomach mesenchyme was shown to require epithelia for it to express smooth muscle actin (Takahashi *et al.*, 1998). This shows that as well as signals from the mesenchyme to the epithelia, there are also signals from the epithelia to the mesenchyme.

The endoderm has been shown to play a role in the development of the mesoderm, heart, head and smooth muscle of the gut. These examples are a reminder that an understanding of endoderm development will be important, not only to understand how the epithelia of the respiratory and digestive systems form, but also to understand the role the endoderm plays in the development of non-endodermal tissues and organs.

1.10 MORPHOGENESIS OF THE ENDODERM

The development of an embryo or organ involves a number of important processes as well as induction and regional specification. Morphogenesis is the process by which the cells of the early embryo produce the shape of the finished organism. There is clearly a massive shape change during the transition from the early endoderm to the organs of the gut and respiratory system. However, little is known about the morphogenesis of the endoderm and in *Xenopus* there are several important aspects of endoderm morphogenesis that are not currently understood.

At neurula stages the *Xenopus* endoderm lines the archenteron cavity (Fig. 1.1A). The dorsal endoderm consists of a single cell layer while the ventral endoderm has several layers of large yolky cells. By stage 45 (5 day old tadpole) the endoderm has undergone enormous elongation and formed the single cell layered epithelium of the intestine. It is not known how the elongation of the endoderm is accomplished. It is also not clear how the thin dorsal layer and thick ventral layers of endoderm combine to produce the single layer of cells that forms the gut epithelium. It is sometimes suggested that the large yolky ventral cells disintegrate and are digested during development (Nieuwkoop and Faber, 1967; Mathews and Schoenwolf, 1998). This implies, although it has not been proven, that that the outer endodermal cells lying closer to the mesoderm form the gut epithelium. Finally, it is not known whether the archenteron cavity of the neurula really gives rise to the gut cavity of the later tadpole. It has been proposed (Goette, 1875), and is generally assumed, that the gut cavity is a continuation of the archenteron. A problem with this model is that the archenteron narrows considerably and appears to close through much of the gut at early tadpole stages. This means that there cannot be a simple transition from archenteron to gut cavity. It has also been suggested, based on vital dye experiments, that the gut cavity is not a continuation of the archenteron at all but opens up *de novo*, as a completely separate cavity, during development (Tahara and Nakamura, 1961). So, although it is widely assumed, it is not known whether the archenteron does in fact give rise to the gut cavity. If the development of the endoderm derived organs is going to be understood then the mechanisms responsible for the

morphogenesis of the endoderm, as well as those responsible for induction and regional specification, need to be established.

Almost nothing is known about the processes responsible for the morphogenesis of the endoderm, so to try and get an understanding of the type of mechanisms that may be involved, it is necessary to look at other systems where the processes that drive morphogenesis are well understood. One system where these processes have been established is gastrulation in *Xenopus*. At the start of gastrulation, the blastopore lip forms on the dorsal side of the embryo (Fig. 1.5A). The blastopore spreads to form a ring round the embryo. During gastrulation, some of the marginal zone ingresses through the blastopore lip and at the end of gastrulation ends up on the inside of the embryo (Fig. 1.5A). This tissue is called the involuting marginal zone (IMZ). Blastula embryos have a two layered structure with a superficial layer of cells covering several layers of deep cells. The superficial layer of the IMZ contains prospective endoderm (yellow in Fig. 1.5) that will form the lining of the archenteron roof. The deep layer contains prospective mesoderm (red) which will form the dorsal axial tissues. Just animal to the IMZ is the non involuting marginal zone (NIMZ) which does not involute during gastrulation and remains on the outside of the embryo (blue, Fig. 1.5A). During gastrulation the IMZ and the NIMZ undergo large changes in shape so that they both become much longer and narrower (Keller, 1976; Keller and Danilchik, 1988; Keller *et al.*, 1985). This simultaneous process of narrowing and elongation is termed convergent extension. The extension of these two tissues can be seen in Figure 1.5A. They are initially quite short but by the end of gastrulation they have extended to cover a large part of the axis. The convergence that occurs simultaneously with the extension reduces the diameter of the marginal zone, which drives the reduction in size and closure of the blastopore that occurs during gastrulation. Therefore, the convergence and extension of the marginal zone is responsible for many of the changes in shape that occur during gastrulation.

If two explants of the marginal zone containing the NIMZ and the IMZ are cut from embryos and placed together, they fuse and form what is called a sandwich explant (Fig. 1.5B). The sandwich explants were found to undergo the same convergent

extension in culture as they normally do during gastrulation (Fig. 1.5B, Keller, 1984; Keller and Danilchik, 1988; Keller *et al.*, 1985). This makes it easy to visualise the extensive convergent extension that these tissues undergo during gastrulation (Fig. 1.5B). It also shows that the ability to undergo convergent extension is an autonomous property of these regions and not caused by an external force.

Having shown that convergent extension is an autonomous property of these explants it was important to establish what was occurring in these tissues to cause this large change in shape. Convergent extension was found to be driven by two cell rearrangements that take place within the tissue (Fig. 1.6). The first cell rearrangement was shown to be a radial intercalation. During radial intercalation cells intercalate perpendicular to the axis of the embryo (Fig. 1.6A). At the start of gastrulation the deep layer is several cells thick. Studies of the layer using electron microscopy showed that early in gastrulation the cells of the deep layer intercalated to produce a single layer of cells (Keller, 1980). This radial intercalation was later visualised in explants of the marginal zone, in real time, using confocal microscopy (Keller *et al.*, 1985). Radial intercalation accounts for some of the extension of the marginal zone during gastrulation. However, radial intercalation will not cause the convergence (Fig. 1.6A) that also occurs in this tissue, so there must be another process involved in convergent extension. Confocal microscopy of explants showed that the cells of these tissues also undergo mediolateral intercalation (Keller *et al.*, 1992b; Keller *et al.*, 1985). During mediolateral intercalation cells rearrange to produce fewer, longer, rows of cells (Fig. 1.6B). Grafts of fluorescently labelled tissue confirmed that this intercalation occurs in intact embryos as well as in explants (Keller and Tibbets, 1989). Mediolateral intercalation will drive both the convergence and extension of the marginal zone. Therefore, two cell rearrangements, radial and mediolateral intercalation, are responsible for the changes in shape that occur during gastrulation.

Cell rearrangements do not just occur during gastrulation in *Xenopus* but are found in a range of other tissues and animals (reviewed in Keller, 1987). For example, radial

Figure 1.5. Convergent extension during gastrulation. A, Convergent extension in the IMZ and NIMZ in whole embryos. B, Convergent extension of the IMZ and NIMZ in sandwich explants. The IMZ is shown in red (future mesoderm) and yellow (future endoderm). The NIMZ is shown in blue. Although it is not discussed here, the sandwich explants also produce differentiated notochord and muscle, as can be seen in the far right panel of B. See Keller *et al.*, 1992a for more details. Figure adapted from Keller *et al.*, 1992a.

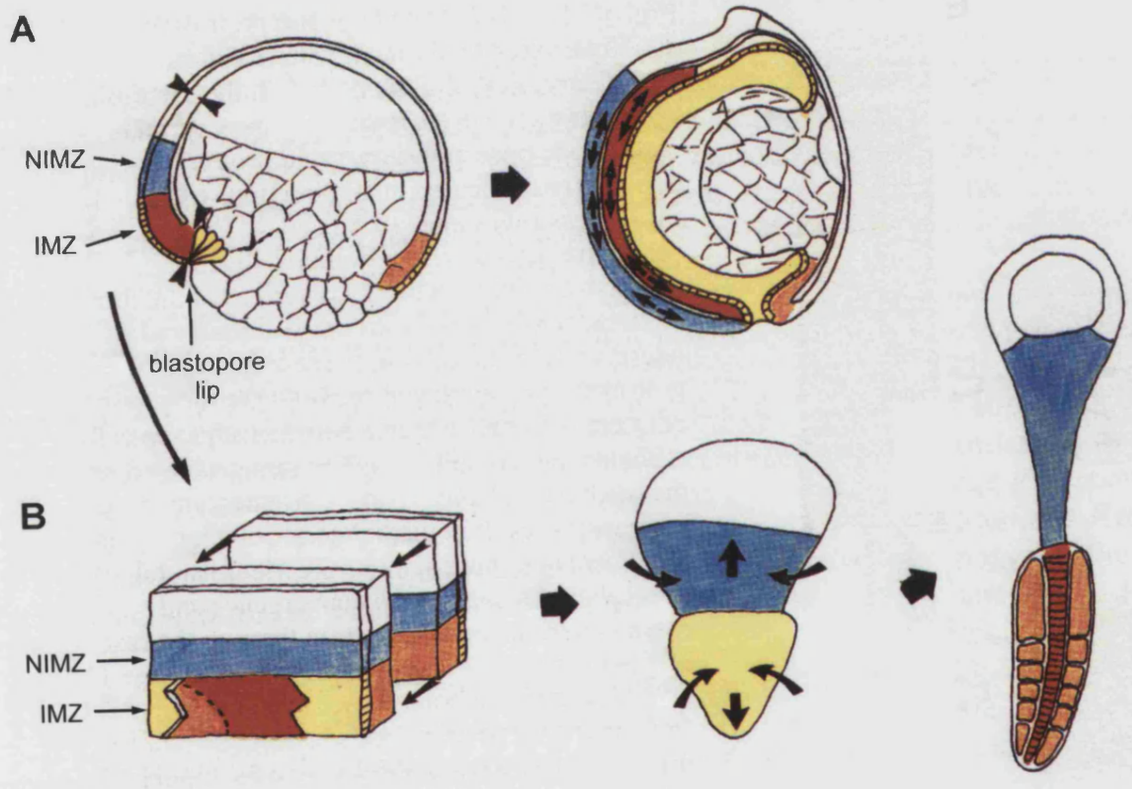
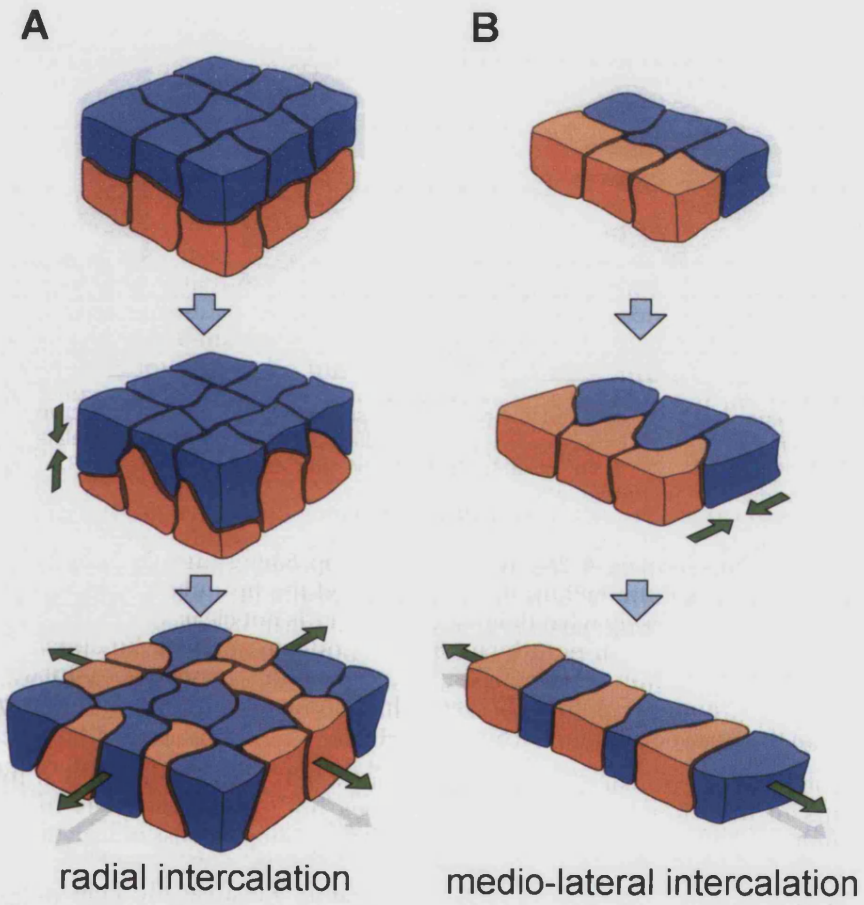

A
NIMZ
IMZ
blastopore
lip
B
NIMZ
IMZ

Figure 1.6. Radial and medio-lateral intercalation. A, radial intercalation. B, medio-lateral intercalation. Figure adapted from Wolpert, 1998.


A
B
radial intercalation
medio-lateral intercalation

and mediolateral intercalation also take place in gastrulating Zebrafish embryos (Warga and Kimmel, 1990) and, in *Drosophila,* cell intercalation has been shown to drive elongation of leg discs (Fristrom, 1976). Therefore, cell rearrangements are a key process in the morphogenesis of a number of systems making it possible that cell rearrangements also play a role in the morphogenesis of the endoderm.

1.11 *XENOPUS* AS A MODEL ORGANISM TO STUDY THE LATER DEVELOPMENT OF THE ENDODERMAL ORGANS

The advantages of *Xenopus* embryos make them a potentially excellent model organism for studying the development of the endoderm. *Xenopus* embryos are large and develop externally making them accessible and easily studied at all developmental stages. Amphibian embryos also heal well after surgery and explanted tissue develops well *in vitro.* This makes *Xenopus* embryos excellent for grafting and tissue explant experiments. The power of explant experiments has already been demonstrated above in the study of the induction and regional specification of the endoderm and in the study of convergent extension during gastrulation.

In *Xenopus* embryos it is possible to carry out gain of function experiments for a gene of interest by the overexpression of injected mRNA (Krieg and Melton, 1984). This approach is very useful for studying early development but has a number of limitations for studying later development. The most important limitation is that injected RNA will be progressively degraded, so the amount of product produced will be gradually reduced. Translation of injected RNA also starts straight after injection so it is not possible to specifically target later development. This is a particular problem where a gene of interest has roles in early and later development as early overexpression may mask an effect on later development. Significantly for the study of later development, many of these problems can be overcome by the use of transgenic *Xenopus* technology (Kroll and Amaya, 1996). The production of embryos with transgenes inserted into their genome means that any gene of interest can be overexpressed by using spatially or temporally specific promoters. Therefore,

there are a number of approaches that make *Xenopus* a very good system for carrying out gain of function experiments.

Xenopus embryos have a very long generation time and are pseudotetraploid, both of which make *Xenopus* a very poor system for genetic studies (Amaya *et al.*, 1998). However, as discussed above, loss of function experiments are made possible in *Xenopus* by using dominant negative receptors (Amaya *et al.*, 1991) and hybrid transcription factors (Conlon *et al.*, 1996). It may also soon be possible to use a close relative of *Xenopus laevis*, *Xenopus tropicalis* to carry out genetic studies (Amaya *et al.*, 1998, throughout this study "*Xenopus*" refers to *Xenopus laevis*). *Xenopus tropicalis* is a true diploid and has a faster generation time than *Xenopus laevis* meaning that *Xenopus tropicalis* has the potential for carrying out genetic studies coupled with the advantages of an amphibian system.

These advantages make *Xenopus* embryos a potentially very attractive model organism for studying not just the induction of the endoderm but all aspects of endoderm development.

1.12 OBJECTIVES OF THIS WORK

Characterisation of the endoderm derived organs in Xenopus. The advantages of *Xenopus* embryos offer great potential for studying the many areas of endoderm development that are not understood. However, a problem in studying the later development of the *Xenopus* endoderm arises from the anatomical complexity of the intestine. A typical transverse section through a young tadpole shows numerous loops of gut and considerable experience is required to identify them or to distinguish between normal and abnormal development. The only available histological atlas (Hausen and Riebesell, 1991) does not extend to the later stages during which gut development occurs. The standard stage series for *Xenopus* does include a written description of gut development but no illustrations (Nieuwkoop and Faber, 1967). In order to carry out any serious study into the development of the endoderm it needs to be possible to identify the organs of the gut and to understand how they are organised during development. To achieve this a comprehensive study of the organisation, cell

types and gene expression of the endoderm-derived organs of the gut and respiratory system was carried out. This study made it possible to carry out the rest of the experiments in this work and should also help many other studies into the later development of the endoderm.

Production of an accurate fate map for the endoderm and gut smooth muscle. The timing and mechanisms responsible for the regional specification of the endoderm remain to be established. It is particularly important to discover how much pattern the endoderm holds after gastrulation and how much of the patterning occurs during the later stages of development. The classical amphibian recombination experiments attempted to address these questions but suffered from several important limitations. The first limitation was that there was no way of showing that the epithelia did not come from contaminating mesoderm. The development of modern cell lineage labels means that this is now possible (Gimlich and Braun, 1985). These experiments also require an accurate fate map. There are a number of existing amphibian endoderm fate maps (particularly Tahara and Nakamura, 1961) but these suffer from the limitations of spreading and fading associated with vital dyes and furthermore none of these older studies were carried out using *Xenopus* embryos. Therefore, a new comprehensive and accurate fate map for the endoderm was produced. This fate map shows which parts of the endoderm give rise to which organs of the gut. The mesenchyme that gives rise to the smooth muscle and connective tissue has been shown to play an important role in the development of the endoderm. Previously it was not known where the smooth muscle originated from in the early *Xenopus* embryo so a second fate map was produced which shows the origin of the gut smooth muscle. These fate maps will make it possible to carry out experiments to look at the specification of the endoderm and smooth muscle and also to investigate the role of the prospective smooth muscle layer in the development of the endoderm.

Establishment of the mechanisms responsible for endoderm morphogenesis. The endoderm fate map shows which parts of the endoderm give rise to which organs of the gut and respiratory system. However, it does not provide answers to the important questions that were not understood about the morphogenesis of the

endoderm. It was not known how the elongation of the endoderm occurs or how the single layered dorsal endoderm and multi layered ventral endoderm give rise to the single layered epithelia of the digestive tract. Finally it was not known if the embryonic archenteron cavity gives rise to the gut cavity. The answers to these questions are fundamentally important to an understanding of the development of the endoderm. Although almost nothing was known about the morphogenesis of the endoderm, investigation of other systems suggests that cell rearrangements could be important in endoderm morphogenesis. In the final part of this project, a number of labelling techniques were used to answer these previously unanswered questions. This work showed that radial intercalation occurs during the formation and elongation of the gut epithelium and that the archenteron almost closes before the true gut cavity opens up from the remnant of the archenteron. Based on these results a new model is proposed that explains the transition from endoderm to epithelium.

CHAPTER 2 MATERIALS AND METHODS

2.1 CARE OF ADULT *XENOPUS*

Adult *Xenopus*, fed on a pellet diet (Blades Biological), were maintained in an aquarium with a 14 hour light/10 hour dark lighting cycle at 21 °C. The week before an experiment females were primed with an injection of 50 units Pregnant Mare Serum Gonadotropin (PMSG, Calbiochem) into the dorsal lymph sac. The evening prior to an experiment females were induced to ovulate with an injection of 300-600 units of Human Chorionic Gonadotropin (Chorulon, Intervet).

2.2 FERTILISATION AND CULTURE OF *XENOPUS* EMBRYOS

In order to allow the *in vitro* fertilisation of eggs a male *Xenopus* was killed using a Home Office approved schedule one procedure (typically overdose in the anaesthetic benzocaine, decapitation and destruction of the spinal cord) and the testis dissected out. The testes were then stored for up to 7 days in NAM solution (Table 1).

Xenopus eggs were collected in petri dishes by exerting gentle pressure on the abdomen of the injected females. A portion of the dissected testis was macerated and spread over the eggs to fertilise them. The eggs were left for approximately 10 minutes and then flooded with sterile Milli-Q water. Approximately 30 minutes later, after rotation had occurred, fertilised eggs were dejellyed in 2.5 % cysteine (pH 7.9), rinsed several times in Milli-Q water and cultured in NAM/10 (Table 1). Staging of embryos was carried out according to the standard stage series for *Xenopus* (Nieuwkoop and Faber, 1967).

2.3 HISTOLOGY

The standard protocol for histology was fixation in Zenkers fixative followed by staining with borax carmine (stains nuclei red) and picroblue black (stains cytoplasm

blue/green). This combination of stains was used as it gave good contrast in all of the tissues examined.

Embryos were fixed in Zenkers fixative (5 g mercuric chloride, 2.5 g potassium dichromate and 1g potassium sulphate per 100 mls of water with 5 % acetic acid added just before use) for 4 hours. The embryos were then washed several times in tap water to remove the dichromate and soaked in Lugol's iodine (1 g iodine and 2 g potassium iodide per 100 mls water) for 30 minutes. The Lugol's iodine removes precipitated mercury.

Table 2.1. Composition of NAM solutions.

	NAM	NAM/2	NAM/10
NaCl	110 mM	55 mM	11 mM
KCl	2 mM	1 mM	0.2 mM
$Ca(NO_3)_2.4H_2O$	1 mM	0.5 mM	0.1 mM
$MgSO_4.7H_2O$	1 mM	0.5 mM	0.1 mM
Sodium EDTA	0.1 mM	0.05 mM	0.01 mM
Sodium Hepes	5 mM	5 mM	5 mM
Sodium Bicarbonate	1 mM	1 mM	-------
Gentamicin sulphate	2.5 µg/ml	2.5 µg/ml	2.5 µg/ml

Following fixation embryos were block stained in borax carmine. Embryos were placed in 10 % borax carmine in 35 % EtOH over night. The embryos were then washed in 70% EtOH/ 1 % HCl until the solution is only slightly pink and left for 4 hours.

After staining the embryos were dehydrated through the following ethanol and Histoclear series, 70 % ethanol, 90 % ethanol, 95 % ethanol, 2 times 100 % ethanol and two times Histoclear. To embed the embryos in wax (Pastillated Lamb Wax,

Lamb) they were heated to 60 °C, placed in 1:1 Histoclear/wax and finally twice in 100 % wax. The wax was then allowed to set in plastic trays.

The wax blocks containing the embryos were trimmed to size and mounted on wooden blocks. A microtome (Jung Biocut, Leica) was then used to cut 8 μm transverse sections of the embryos which were placed on subbed slides and dried over night on a 37 °C hot plate. Slides were subbed by washing in hot soapy water, rinsing in Milli-RO water, rinsing in 95 % EtOH/0.1 % acetic acid and allowing them to dry. The slides were then dipped for one minute in 2 % 3(triethoxysilyl)-propylamin (APTS, Sigma) in acetone (carried out in a fume hood), dipped in acetone, rinsed in Milli-RO water and finally allowed to dry at 37 °C.

Sections were then hydrated, stained with picroblueblack (5 g of wet Picric acid was added to 480 mls of water, filtered, and then mixed with 20 mls of 0.5 % naphthalene black in water), dehydrated and mounted in DPX (BDH) using the following sequence 2 x 2 minutes Histoclear, 2 x 1 minute 100 % EtOH, 1 min 95 % IMS, 1 min 90 % IMS, 1 min 70 % IMS, 1 min 50 % IMS, 1 min water, 1 min picroblueblack, 1 min water, 1 min 70 % IMS, 1 min 90 % IMS, 1 min 95 % IMS, 2x1 minute 100 % EtOH, 2 x 1 minute Histoclear and finally DPX.

Photographs of histological sections were taken using a colour CCD camera (Hamamatsu), imported into Adobe Photoshop and printed on a dye sublimation printer (Kodak) or an inkjet printer (Hewlett Packard).

2.4 DRAWING SECTIONS OF THE *XENOPUS* GUT

Sections from different positions along the anterior/posterior axis were drawn for 3, 4, 5, 6 and 7 day old (stages 40/41, 44/45, 45/46, 46 and 46/47) embryos with the aid of a drawing tube (Leica) attached to a dissecting microscope (Wild M8, Leica). At least six embryos, coming from at least 3 different batches of embryos, were drawn for each day of development. A representative case was chosen for each time point and the drawings from these embryos were scanned into a computer and then shaded and labelled using Adobe Photoshop.

2.5 PREPARATION OF DISSECTED GUTS

Xenopus embryos at 3 days of development (stage 40/41) were fixed whole for 45 minutes while 5 and 7 days post fertilisation embryos (stage 45/46, 46/47) were killed by an overdose of the anaesthetic MS222 (1/200 w/v). The outer membranes surrounding the gut were removed using fine pointed forceps. The liver was then separated from the anterior body wall and the exposed gut was removed with forceps, washed in NAM/10 (MS222 precipitates in the presence of formaldehyde) and placed in fixative (10 % formalin, 70 % PBS). While in fixative, the gut was uncoiled by placing a fragment of glass coverslip on the posterior end, pulling the anterior end and then placing a second fragment of coverslip on the anterior end. The guts were left to fix overnight and then stored in 70 % ethanol. Photographs of the dissected guts were taken using a conventional camera mounted on a dissecting microscope (Wild M8, Leica), scanned into Adobe Photoshop and printed out as above.

Embryos for whole mount drawings were fixed as above, the membranes surrounding the gut removed using forceps and then drawn and processed as for the sections.

2.6 WHOLEMOUNT *IN SITU* HYBRIDISATION

Messenger RNA (mRNA) was visualised in wholemount guts using standard wholemount *in situ* hybridisation procedures (Harland, 1991) with modifications from (Pownall *et al.*, 1996) whereby a digoxigenin (DIG) labelled anti-sense probe binds specifically to the endogenous message. Alkaline phosphatase coupled to an antibody specific for DIG is then used to drive a colour reaction that produces a blue precipitate where the RNA of interest is expressed.

Synthesis of DIG labelled probe. A labelling reaction was set up at room temperature containing 10 µl of 5x transcription buffer (Promega), 2.5 µl of 10x DIG labelling mix (Boehringer Manheim), 5 µl of 100 mM Dithiothreitol (DTT), 2 µl of RNasin (50 units, Promega), 3 µl of appropriate polymerase (see below), 2.5 µg of linear DNA template and made up to 50 µl with DEPC treated water. DEPC water

was prepared by adding 0.1 % DEPC (v/v) to Milli Q water which was allowed to stand overnight and then autoclaved. DEPC should be handled in a fume cupboard.

The reaction mixture was then incubated at 37 °C for 2 hours when an extra 1 µl of polymerase was added and the reaction mixture left for a further 2 hours. The DNA template was then removed by treatment with 2.5 µl RNase free DNase 1 (Promega) for 20 minutes at 30 °C and the RNA synthesis checked by running a 2 µl aliquot on a 1.5 % agarose (Gibco) gel. Agarose gel electrophoresis was carried using standard procedures (Manitias *et al.*, 1982).

The RNA was then precipitated with lithium chloride (final concentration 2.5M) overnight at -20 °C. The RNA was then centrifuged (13,000 rpm in a microcentrifuge, Sanyo) for 10 minutes and the RNA pellet washed in 70 % ethanol and resuspended in DEPC water with 1 mm EDTA. The yield was then checked by running a sample on a 1.5 % agarose gel and the rest of the RNA probe stored at -80 °C.

The probes (along with the enzyme used to linearise the template DNA, the RNA polymerase used and the size of the RNA probe) used were *Endodermin* (EcoR1/T7, 4 kb), *XlHbox8* (Not1/T3, 270 bp), *IFABP* (Xho1/T7, 288 bp), *Xsox2* (EcoR1/T7, 1.4 kb), *Xcad1* (Not1/T7, 500 bp) and *Xcad2* (Not1/T7,1 kb). After the plasmid was linearised with the appropriate enzyme, it was isolated using Gene Clean (Bio 101) and used as described above.

Preparation of guts for in situ. Guts were dissected as described above except that they were fixed in MEMFA (0.1M MOPS (pH 7.2), 2mM EGTA, 1mM $MgSO_4$ and 10 % (v/v) formalin. pH 7.4) for 1 hour. After fixation the guts were washed in 100 % ethanol, placed in fresh 100% ethanol and stored at -20 °C.

In situ protocol. Unless otherwise stated all washes were carried out in scintillation vials at room temperature on a roller. Dissected guts were brought to room temperature washed in 75 % ethanol/ PBST (PBS+ 0.1% Tween 20) for 10 minutes and then 50 % ethanol/PBST for 10 minutes. The guts were then washed 3 times in PBST for 5 minutes and treated with 10 µg/ml protease K for 10 or 20 minutes. The

guts were not placed on a roller during protease K treatment but the solution was mixed gently. The guts were then washed in triethanolamine (0.1M, pH 7.8) for 5 minutes. They were then washed again in triethanolamine with 12.5 µl of acetic anhydride added. After 5 minutes another 12.5 µl of acetic anhydride was added and the mixture left for a further 5 minutes. The acetylation treatment reduces non specific background in the *in situs*. The guts were then washed twice in PBST for 5 minutes, refixed for 20 minutes in 10 % formalin (v/v) in PBST and washed 5 times in PBST for 5 minutes. The guts were then placed in 1 ml of PBST and 250 µl of hybridisation buffer.

The hybridisation buffer consisted of 50 % Formamide (v/v, Amresco), 5x SSC (1x SSC contained 3M NaCl, 0.3M Nacitrate.$2H_2O$, pH 7), 1 mg/ml yeast total RNA (ICN Biomedicals), 100 µg/ml heparin, 1x Denhart's (10 g Ficoll 400, 10 g polyvinylpyrrolidone, 10 g BSA in 500mls), 0.1% Tween 20 (v/v), 0.1% CHAPS (w/v), 10 mM EDTA made up in DEPC treated water. Formamide and solutions containing formamide should be handled with caution. The guts were then placed in fresh hybridisation solution, transferred to 1.5 ml tubes and placed at 60 °C for 10 minutes. The hybridisation solution was then replaced with fresh solution (warmed to 60 °C) and placed in a hybridisation oven at 60 °C and agitated gently for at least 2 hours. The hybridisation solution was then replaced with 60 °C hybridisation solution with 1 µg/ml probe added and agitated gently over night in a 60 °C hybridisation oven.

The next day the probe was removed (The probe can be kept at -70 °C and re-used) and the guts washed twice in hybridisation solution at 60 °C for 10 minutes. While changing solutions the tubes were kept in a hot block to keep the specimens at 60 °C. The guts were then washed three times in 2x SSC + 0.1% Tween 20 for 20 minutes at 60 °C. The guts were then washed three times 0.2 % SSC + 0.1% Tween 20 for 30 minutes at 60 °C. The guts were washed twice in Maleic acid buffer (MAB, 100 mM Maleic acid, 150 mM NaCl, 0.1 % Tween 20, pH 7.8) for 15 minutes at room temperature and then incubated with MAB + 2 % Boehringer Mannheim Blocking reagent for 30 minutes. Then the guts were pre incubated with MAB + 2 % Boehringer Mannheim Blocking reagent (v/v) and 20 % heat treated lamb serum

(v/v) and rocked for 2 hours at room temperature. After two hours, fresh MAB + 2 % Boehringer Mannheim Blocking reagent (v/v) and 20 % heat treated lamb serum (v/v) was added with 1/2000 sheep anti digoxigenin antibody coupled to alkaline phosphatase (Boehringer Manheim) and left overnight on a roller at 4 °C.

The following day the guts were washed 3 times in MAB for 5 minutes. The guts were then transferred back to scintillation vials and washed 3 times 1 hour in MAB. They were then washed twice in alkaline phosphatase buffer (100 mM Tris (pH 9.5), 50 mM $MgCl_2$, 100 mM NaCl, and 0.1% Tween 20) +5 mM levamisole and the alkaline phosphatase activity detected by incubation with BM purple precipitating alkaline phosphatase detection system (Boehringer Mannheim) + 5mM levamisole. The levamisole inhibits endogenous alkaline phosphatase activity. The colour reaction was stopped by washing twice in PBST and the guts were stored in MEMFA.

2.7 STAINING OF ENDOGENOUS ALKALINE PHOSPHATASE ACTIVITY

Guts were dissected as above except for the fixation which was in MEMFA for 1 hour and then stored at -20 in MeOH. The guts were then washed 3 times 5 minutes in PBST, treated for 10 minutes with 10 µg/ml protease K and rinsed twice with PBST. They were then washed 3 times in alkaline phosphatase buffer and the alkaline phosphatase activity detected by incubation with BM purple precipitating alkaline phosphatase detection system (Boehringer Mannheim).

2.8 FATE MAPPING WITH FDA

Injection of fluorescent label. Injections were carried out using a Drummond micro injector with needles pulled from Drummond 3.5" capillary tubes using a needle puller (SRI, 2001). Xenopus embryos at the 2 cell stage were placed in NAM supplemented with 5 % ficoll and each blastomere injected with 0.25 ng (4.6 nl of 50 mg/ml) of lysine fixable Fluorescein Dextran Amine (FDA) (Molecular Probes, D1820). Injected embryos were left in NAM/ficoll until they reached blastula stages

at which point they were placed in NAM/10 supplemented with 5 % ficoll. The ficoll is added to shrink the vitelline membrane on to the embryo and to encourage healing. The NAM supplemented with ficoll is replaced with NAM/10 supplemented with ficoll as the embryos will exogastrulate if left in NAM.

Grafting. Stage 13/14 (early neurula) embryos were placed in NAM/2 and their Vitelline membranes removed using sharpened forceps (No5s, Aldrich). A rectangular piece of tissue containing all three germ layers, was cut from an unlabelled host embryo at one of 14 standard positions (see results). Tissue was cut from embryos using tungsten needles mounted in capillary tubing. The needles were sharpened by electrolysis with a direct current in 1 M NaOH. A piece of tissue was then taken from a labelled donor embryo at the same position and placed in the hole left in the unlabeled host embryo. A glass square cut from a coverslip was placed over the host embryo for approximately 40 minutes to hold the graft in place and allow healing to occur. After healing the cover slip was removed, the embryos were placed into individual agar coated wells of a 24 well dish and left overnight. Next morning the NAM/2 was replaced with NAM/10 and the embryos left to develop.

Scoring the embryos. After 1 week of development embryos that developed normally were fixed for 24 or 48 hours in formalin (10 % formalin in 70 % PBS) and dehydrated through a BuOH series (70 % IMS, 80 % IMS, 80 % IMS:BuOH (3:1), 90 % IMS:BuOH (1:1), 100 % IMS:BuOH (1:3) and 100 % BuOH. Specimens dehydrated through BuOH seemed to have lower background fluorescence than those dehydrated through an ethanol series. After dehydrating the specimens were embedded in paraffin wax and sectioned as previously described for histology. Sections were not counter stained so after sectioning they were mounted in DPX straight after dewaxing in Histoclear. FDA in the mounted sections was then visualised using fluorescent light from a mercury lamp attached to a compound microscope (DMRB, Leica). Photographs of the FDA were taken using a conventional camera with Kodak film (Elite Chrome, ISO 400). Sections from embryos that were chosen as typical individuals were drawn with the aid of a drawing tube (Leica) and the position of FDA label added to the diagrams. A small

number of the mesoderm cases were scored in whole mount guts rather than in sections. In these cases the graft were carried as above except that the graft contained only ectoderm and mesoderm. The embryos were then dissected and the label visualised as described below for DiI labelling.

2.9 FATE MAPPING WITH DiI

To specifically label small populations of endoderm cells a fixable derivative of the lipophilic dye DiI was used (Cell Tracker CM-DiI, Molecular Probes, referred to simply as DiI). This was dissolved at 3 mg/ml in ethanol + 100 mg/ml phosphatidylcholine, heated to 50 °C and diluted 1/10 in 0.2 M sucrose at 50°C. The DiI was then centrifuged to remove any precipitate and loaded into a needle mounted on a Drummond microinjector. The microinjector was then used to fire a pulse of 4.6 nl of DiI at the cells being labelled. Stage 14 embryos were labelled with DiI at one of 4 positions (see chapter 7).

Embryos labelled with DiI at stage 14 were fixed at stage 14 and 39/40, embedded, sectioned and scored as previously described for the FDA labelling. DiI labelled embryos were also scored at Stage 45 as isolated gut preparations. They were dissected as described above and the DiI was then visualised using a fluorescent dissecting microscope (Leica). Confocal images were then captured using a Zeiss 510 laser scanning microscope. DiI + FDA were collected with the 458 nm line (argon) and 343 line (HeNe) lasers and superimposed on a transmitted light image (458 nm).

2.10 FATE MAPPING WITH FDA + DiI

FDA labelled donor and unlabelled host embryos were prepared as above. Regions of the endoderm were then labelled with DiI as described above and by grafts of FDA labelled tissue. The combinations of positions that were double labelled are described in chapter 7. The double labelled embryos were then cultured, dissected and scored as described for the DiI labelling.

2.11 BIOTIN LABELLING

The entire external surface of late blastulae (stage 9/10) embryos was labelled using a slightly modified version of a previously described method (Minsuk and Keller, 1997). Dejellied embryos were placed, with their vitelline membranes intact, into 1.5 mls of sulfo-NHS-LC-biotin (Pierce), 5 mg/ml in NAM/10 for 5 minutes. The labelling solution was prepared immediately before use to minimise hydrolysis of the reagent. The embryos were then washed 3 times for 5 minutes in glycine (10 mM in NAM/10), to quench the reaction, and cultured in NAM/10. At the required stage embryos were fixed in MEMFA for 2 hours, dehydrated through an ethanol/Histoclear series and embedded in paraffin wax (as described above). The embryos were then sectioned, dewaxed in Histoclear and hydrated through an ethanol series (as described above). The sections were then placed in Tris buffered saline (TBS: 100 mM TRIS pH 7.4, 50 mM $MgCl_2$, 100 mM NaCl, 0.1 % Tween 20) for 5 minutes and were blocked for 1 hour by placing in TBS + 1 % BSA. Slides were then placed horizontally in a humid chamber on plastic supports. While in the chamber 0.75 mls of each solution was used to cover the slides. Slides were twice covered with alkaline phosphatase-conjugated streptavidin (Vector Labs) diluted 1:500 in TBS + 1 % BSA for one hour. The slides were placed back in racks and washed 3 times in TBS for 15 minutes. They were then placed back in the horizontal chamber and were rinsed in alkaline phosphatase buffer and then covered in fresh AP-buffer for 5 minutes. The colour reaction was then carried out by covering the slides in BM purple (Roche) + 5 mM levamisole. The reaction was then stopped by immersing the slides in TBS + 5 mM EDTA for 20 minutes, the slides dehydrated through an ethanol series and mounted in DPX (BDH). Images were recorded using a colour CCD camera (Hamamatsu).

CHAPTER 3 THE TADPOLE GUT AND RESPIRATORY SYSTEM

3.1 INTRODUCTION

Xenopus embryos are potentially an excellent model organism for investigating the mechanisms of endoderm development (discussed in chapter 1.11). *Xenopus* embryos are accessible at all developmental stages and it is possible to investigate the role of a gene by gain or loss of function experiments. However, a problem in studying the later development of the *Xenopus* endoderm arises from the anatomical complexity of the intestine. A typical transverse section through a young tadpole shows numerous loops of gut making it very difficult to identify individual organs or to distinguish between normal and abnormal development. The only available histological atlas (Hausen and Riebesell, 1991) does not extend to the later stages during which gut development occurs. The standard stage series for *Xenopus* does include a written description of gut development but no illustrations (Nieuwkoop and Faber, 1967). Until this problem of identification was overcome, no serious investigation of gut development was possible.

To solve this problem a comprehensive study of the anatomy and histology of the organs of the developing *Xenopus* gut (including the pancreas, liver and gall bladder) was carried out. The study starts at 3 days of development when the gut is a fairly simple tube consisting of undifferentiated cells and finishes at 7 days of development by which time the gut has formed a complex coiled structure with each organ containing clearly differentiated cell types. The changes in the 3 dimensional organisation of the gut during development and the histology of the cells in each organ are described. The organs and cells of the respiratory system, another major derivative of the endoderm, are described in the last part of this chapter.

3.2 THE THREE DIMENSIONAL ORGANISATION OF THE *XENOPUS* TADPOLE GUT

In the following description the terms "anterior, posterior, dorsal and ventral" are used to used to indicate a position in space, while "proximal-distal" is used to indicate position along the length of the gut. The pharynx is at the proximal end and the proctodaeum at the distal end.

In order to understand how the organs of the *Xenopus* gut are arranged in three dimensions, transverse sections of 3, 4, 5, 6, and 7 day old (stage 40/41, 44/45, 45/46, 46, 46/47) embryos were prepared. Sections from the anterior to the posterior were then drawn allowing the path of the gut to be followed through the body. The organs in the drawings were then identified based on their histology and the written description of gut development (Nieuwkoop and Faber, 1967). These identifications were also confirmed by looking at enzymatic activity and gene expression patterns in wholemount tadpole guts (described in chapter 4).

At least six embryos from at least 3 different batches of eggs were drawn for each time point. This showed that although complex, the coiling of the gut was reproducible. Within a batch of embryos there was some variation between individuals, however, this variation was caused by variation in the rate of development and not in the actual anatomy. Based on the drawings of sections from a representative embryo for each time point, a series of schematic diagrams were produced. These diagrams show a ventral view of the organisation of the gut and how this organisation changes during development (Fig. 3.1A-D). In order to increase the clarity of the schematic diagrams, the gaps between parts of intestine and the size of some of the loops has been exaggerated. However, these diagrams are a reliable guide to the organisation of the gut.

After 3 days of development (Fig. 3.1A) the oesophagus (oe) runs posteriorly from the pharynx (a chamber common to both the gut and the respiratory system) in a dorsal position until it reaches the stomach (st). As the oesophagus meets the stomach it turns ventrally and leftwards. The stomach is located on the left hand side

of the embryo in a dorsal ventral orientation. The ventral end of the stomach merges with the large mass of the intestine. The prospective liver (lv), gall bladder (gb) and pancreas (pa) lie in the kink of the gut formed by the oesophagus, stomach and intestine (i). The liver and gall bladder are more anterior and the pancreas more posterior and leftwards.

By the fourth day of development (Fig. 3.1B) the intestine has greatly elongated forming a looped structure. As before, the oesophagus runs posteriorly in a dorsal position until it turns ventrally to meet the stomach. The stomach has moved from the left of the embryo to a more central position although it is still orientated dorsal/ventrally. After the body of the stomach, there is a region of the gut, called the transitional zone (tz), which leads anteriorly for a short distance. After the transitional zone the gut loops back and heads posteriorly in a more dorsal position. This posterior heading section is the first region of the small intestine (labelled sia, the labelling of the intestine is explained below). The liver and gall bladder are located in an anterior/ventral position adjacent to the pharynx and oesophagus and immediately posterior to the heart. Ducts from the posterior of the liver and anterior of the pancreas merge to form the common bile duct which enters the intestine in section ia. The pancreas is located posteriorly to the liver between two loops of intestine and the stomach. This organisation of the oesophagus, stomach, liver, pancreas and first part of the intestine is maintained through the next 3 days of development.

At the other end of the gut the intestine runs from the proctodaeum (pr) in a dorsal anterior direction to a point that is anterior to the stomach before turning and heading posteriorly (id and ib3). This places a distal part of the intestine in close proximity to the oesophagus. Like the anterior parts of the gut discussed above, this posterior part of the intestine keeps this organisation over the next three days of development. While the organisation of the anterior and posterior regions of the gut are maintained, the intestine between these two parts continues to coil.

After 5 days of development (Fig. 3.1C) it becomes apparent that the intestine is forming a double coiled structure. A loop is referred to as a 360 ° turn of intestine and a coil as more than one loop of intestine. As in a four day old embryo the transitional zone runs dorso/anteriorly from the body of the stomach. The gut then turns and first section of the small intestine (sia) runs parallel to the transitional zone in a posterior direction. It is after this posterior heading section that the first of the two coils starts. The first coil is a posterior/ventral/left facing external coil that loops twice anticlockwise (sib1-sib4). At the end of the first coil the intestine starts to loop clockwise forming a smaller anterior/dorsal facing internal coil (sic1-lic5). At this stage of development the external and internal coils both have two loops. At the end of the second coil the intestine turns and runs posteriorly along the dorsal wall of the body cavity finally heading ventrally to the proctodaeum. At this point in development it is possible to see the increase in width that marks the boundary between the small and large intestine. While this double coiled structure is being produced by the intestines, the organisation of the proxiamal and distal sections of the gut is maintained. However, one change that does occur is that the base of the stomach becomes displaced to the right. This displacement means that the stomach is now orientated diagonally from a dorsal middle position to a ventral right position.

There is little change in the layout of the gut from the 5th to the 7th day of development except for an increase in the number of loops in each coil. By the 7th day of development (Fig. 3.1D) the external coil has 3 loops and the internal coil 2 full loops and two kinks. The two coils of intestine have also shifted more into the middle of the body and so no longer face leftwards. This is easier to see in the whole mount drawings (Fig. 3.1G-H).

To help the identification and description of individual loops a new nomenclature system was devised. The first region of intestine that leads posteriorly is suffixed a. Distal to this the first region of the external coil is labelled b1, the next section b2 and so on until the internal coil is reached. The regions of the internal coil are suffixed c instead of b and numbered as above. The last section of the intestine that leads straight posteriorly to the proctodaeum is suffixed d. Where the small intestine

can be distinguished from the large intestine these suffixes are added to si and li respectively. At four days of development it is not possible to distinguish small and large intestine so they are added to i for intestine. This nomenclature system gives each section of the intestine an anatomical and a positional label.

As a complement to the schematic diagrams and to display which parts of the gut are visible from the outside of the embryo, whole embryos were drawn at the same developmental time points and labelled using the same nomenclature system (Fig. 3.1E-H). In order to see the gut more clearly the pigmented epithelium that surrounds it was removed. With the skin and coelomic lining epithelium surrounding the gut removed, at least part of the stomach, small intestine, liver, pancreas and proctodaeum can be seen. The organs that cannot be seen are the oesophagus and large intestine.

Some of the drawings of the sections used for the construction of the schematic diagrams are shown for 3, 4, and 5 days of development (Fig. 3.2, 3.3 and 3.4). These drawings are comprehensive, to scale and labelled with the same system used for the schematic diagrams. They also include a wholemount drawing of a *Xenopus* embryo (from figure 3.1) showing the approximate position of the top section in each column and the last section (marked with *). These figures mean that any piece of gut seen in a section of a *Xenopus* embryo can be identified and its relative position along the length of gut easily established by referring back to the schematic diagrams. Therefore, these drawings provide an atlas for working with sections of the *Xenopus* gut.

3.3 HISTOLOGY OF THE *XENOPUS* TADPOLE GUT

Having described the gross anatomy of the developing *Xenopus* gut, the histology of the cells of each organ will now be described. The histological stains used throughout this study were borax carmine and picroblue black because they gave good contrast in all of the gut tissues. As discussed above, the mesoderm-derived tissues, such as

Figure 3.1. Schematic and wholemount drawings of the *Xenopus* gut. A-D, schematic diagrams. A, 3 day old embryo, B, 4 day old embryo, C, 5 day old embryo, D, 7 day old embryo. The schematic diagrams are from a ventral view with anterior at the top and posterior at the bottom. The right of the gut is on the left of the page and *vice versa*. E-H, drawings of the guts of whole *Xenopus* embryos after the pigmented epithelium has been removed. Starting from the left, each drawing has a right, a ventral and a left view with anterior at the top. E, 3 day old embryo, F, 4 day old embryo, G, 5 day old embryo, H, 7 day old embryo. The gut has been shaded dark grey. The pancreas and liver have been shaded light grey. Scale bar, 1 mm. oe, oesophagus, lv, liver, gb, gall bladder, pa, pancreas, i, intestine, si, small intestine, li, large intestine st, stomach, pr, proctodaeum, ht, heart, tz, transitional zone. The first section of the intestine is suffixed a. Sections of the external coil of intestine are suffixed b1, b2 etc while sections of the internal coil are suffixed c1, c2 etc. The final section of the intestine leading to the proctodaeum is suffixed d (See text for full description of intestine nomenclature).

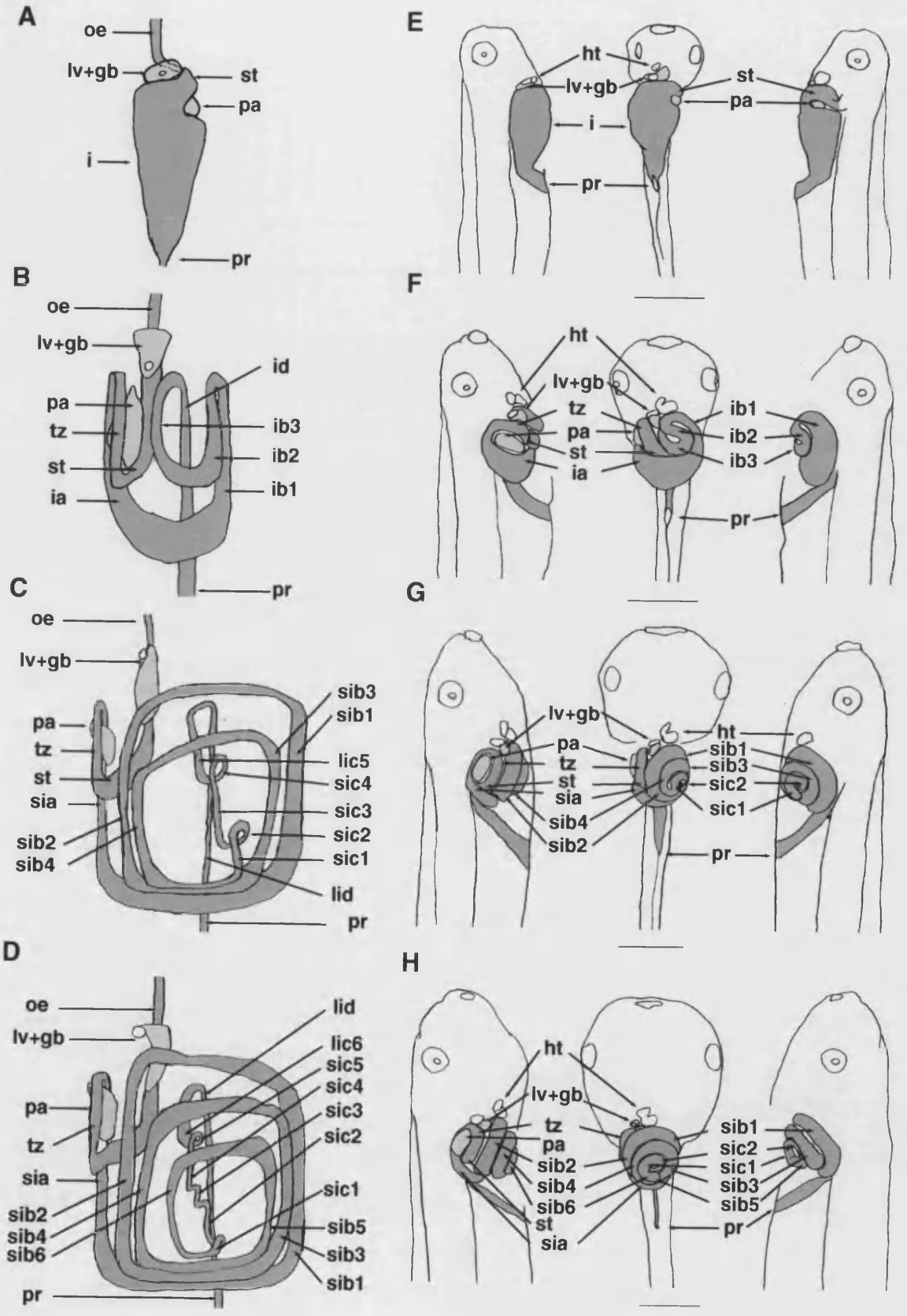

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Figure 3.2. Drawings of transverse sections of a 3 day old *Xenopus* embryo. Each drawing has dorsal at the top and ventral at the bottom. The top left drawing is the most anterior section while the bottom right drawing is the most posterior section. A wholemount drawing is included showing the position of sectioning for the top drawing in each column and the last drawing (*). The gut has been shaded dark grey. The pancreas and liver have been shaded light grey Scale bar, 1 mm. CNS, central nervous system, no, notochord, ms, muscle, ht, heart, ph, pharynx, lv, liver, gb, gall bladder, st, stomach, oe, oesophagus, tr, trachea, lu, lungs, pa, pancreas, i, intestine, pr, proctodaeum, nd, nephritic duct.

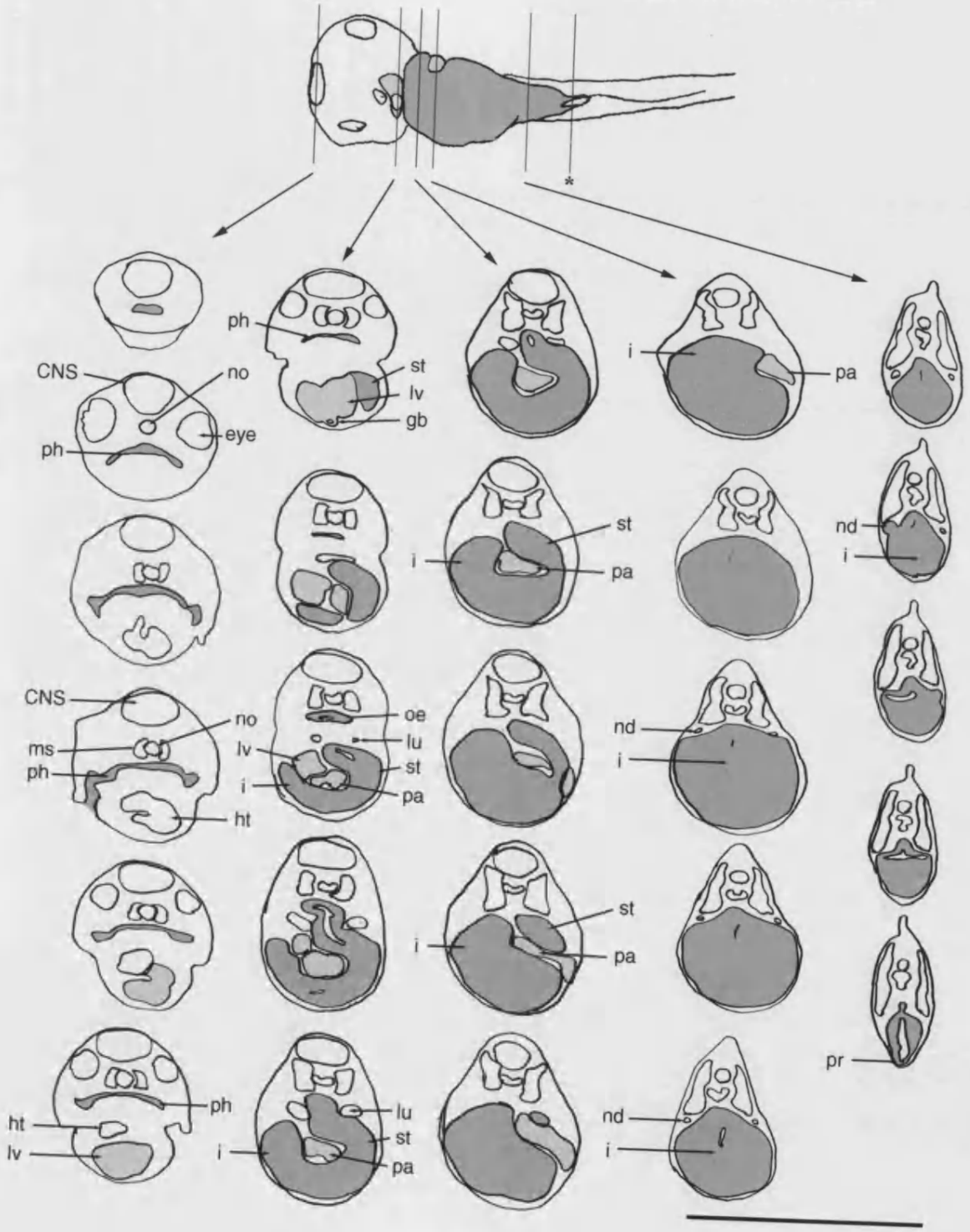
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Figure 3.3. Drawings of transverse sections of a 4 day old *Xenopus* embryo. Each drawing has dorsal at the top and ventral at the bottom. The top left drawing is the most anterior section while the bottom right drawing is the most posterior section. A wholemount drawing is included showing the position of sectioning for the top drawing in each column and the last drawing (*). The gut has been shaded dark grey. The pancreas and liver have been shaded light grey Scale bar, 1 mm. CNS, central nervous system, no, notochord, ms, muscle, ht, heart, ph, pharynx, tn, tongue, lv, liver, gb, gall bladder, st, stomach, oe, oesophagus, tr, trachea, lu, lungs, pa, pancreas, i, intestine, pr, proctodaeum, nd, nephritic duct, bd, bile duct, ov, otic vesicle, tz, transitional zone. The first section of the intestine is suffixed a. Sections of the external coil of intestine are suffixed b1, b2 etc. The final section of the intestine leading to the proctodaeum is suffixed d (See text for full description of intestine nomenclature).

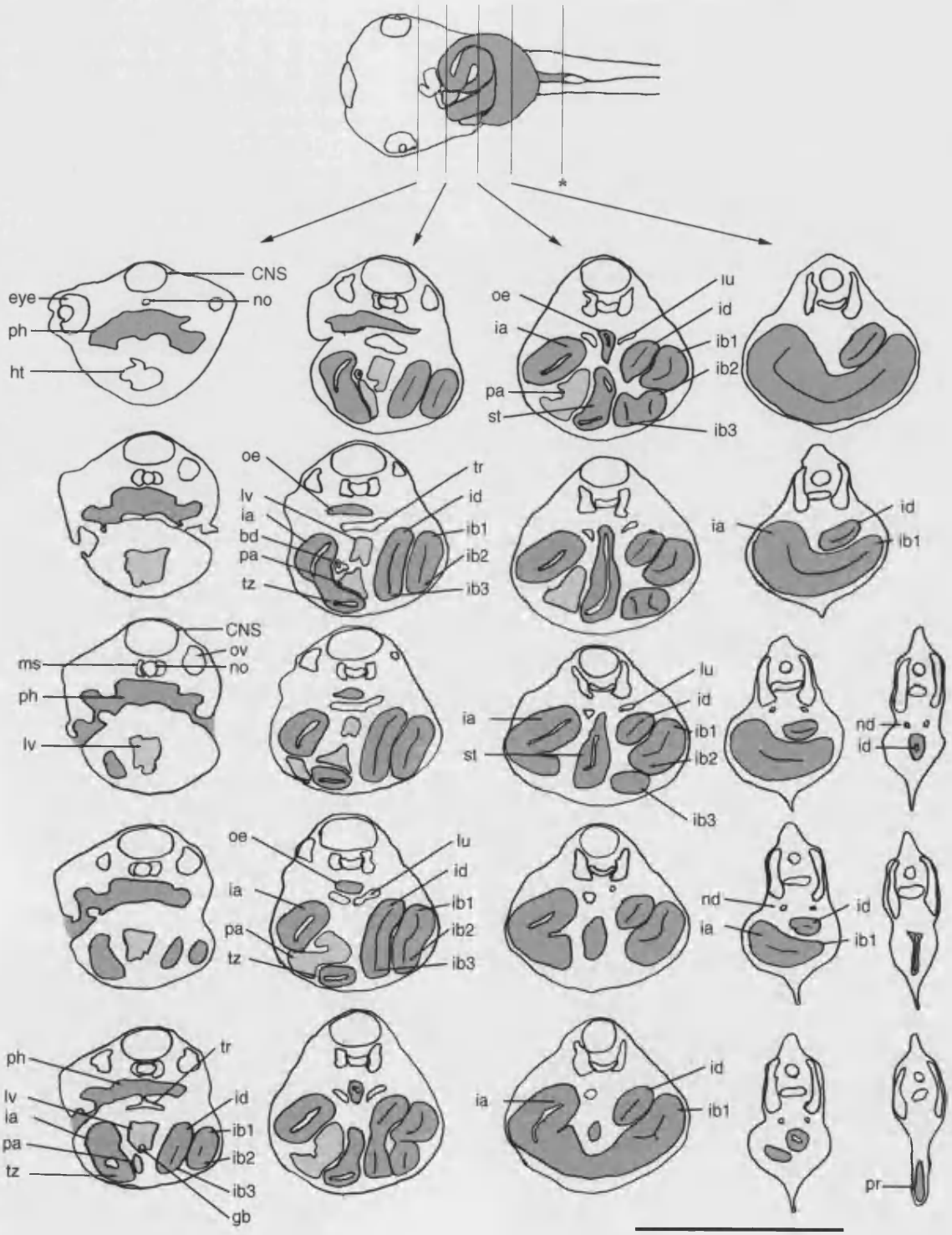
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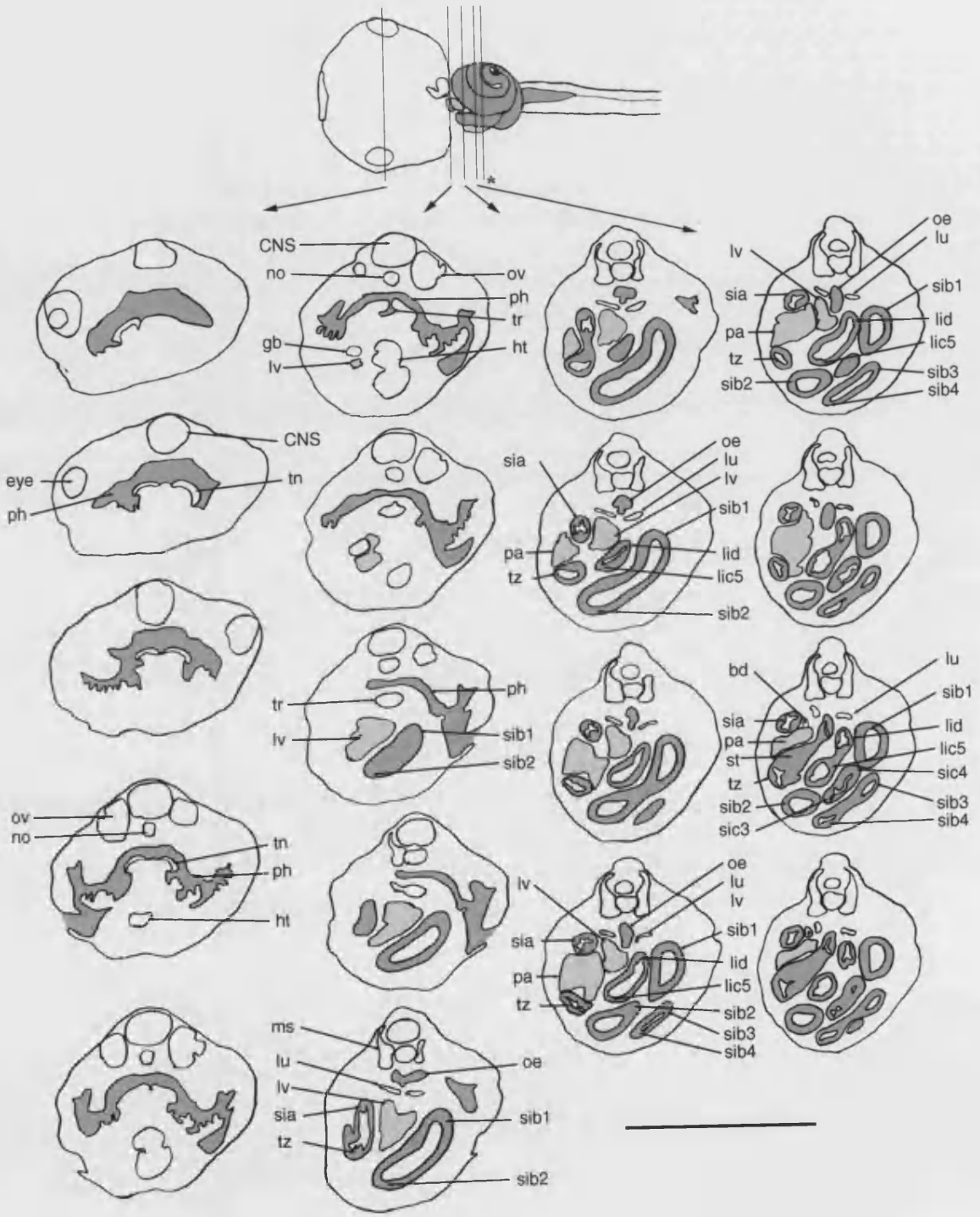
Figure 3.4. Drawings of transverse sections of a 5 day old *Xenopus* embryo. Each drawing has dorsal at the top and ventral at the bottom. The top left drawing is the most anterior section while the bottom right drawing is the most posterior section. A wholemount drawing is included showing the position of sectioning for the top drawing in each column and the last drawing (*). The gut has been shaded dark grey. The pancreas and liver have been shaded light grey Scale bar, 1 mm. CNS, central nervous system, no, notochord, ms, muscle, ht, heart, ph, pharynx, tn, tongue, lv, liver, gb, gall bladder, st, stomach, oe, oesophagus, tr, trachea, lu, lungs, pa, pancreas, si, small intestine, li, large intestine, pr, proctodaeum, nd, nephritic duct, bd, bile duct, ov, otic vesicle, tz, transitional zone. The first section of the intestine is suffixed a. Sections of the outside coil of intestine are suffixed b1, b2 etc while sections of the inside coil are suffixed c1, c2 etc. The final section of the intestine leading to the proctodaeum is suffixed d (See text for full description of intestine nomenclature).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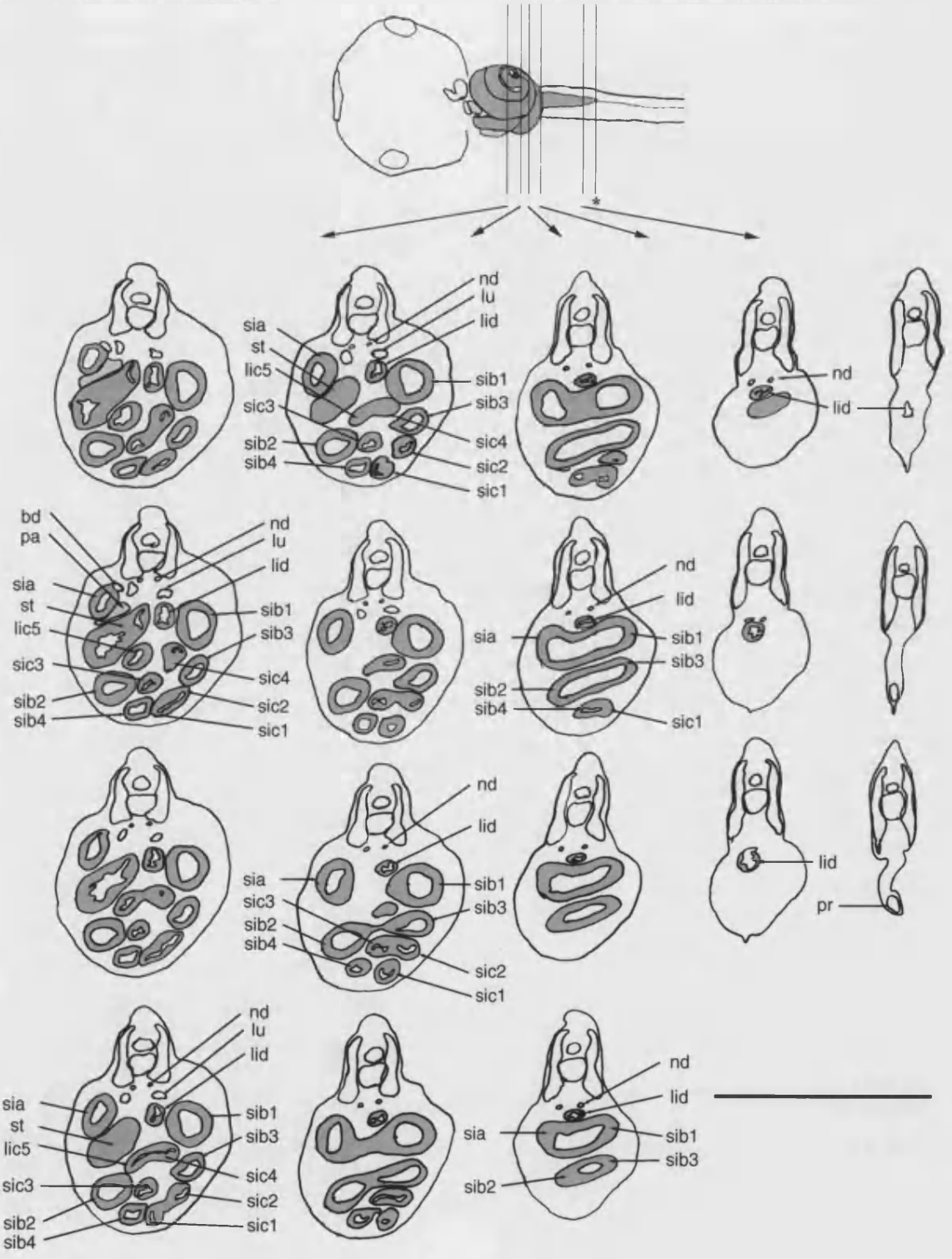
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smooth muscle and connective tissue, are known to play a role in the development of the epithelia. For this reason the histology of the mesoderm-derived tissues as well as the endoderm-derived tissues will be described.

After 3 days of development the cells of the gut showed little differentiation and contained large numbers of yolk granules. Two days later, five days after fertilisation, some of the cells of the gut have begun to differentiate and the amount of yolk granules is reduced. By the seventh day of development each organ of the gut contains clearly differentiated cell types with very few yolk granules remaining from the egg. The organs of the gut are shown at low magnification (Fig. 3.5A-F) for identification and the histology described using higher magnification (Fig. 3.5G-S).

Pharynx. After seven days of development the epithelium of the pharynx contains at least two clearly distinguishable tissue types. The cells of the roof of the pharynx are small and spread out in a squamous epithelium (Fig. 3.5G). Clearly visible on the floor of the pharynx is the tongue, whose cells form an elongated columnar epithelium with their nuclei close to the external surface (Fig. 3.5H). The mesodermal tissue around the pharynx is a loose packed mesenchyme (m) (Fig. 3.5G+H).

Oesophagus. Distal to the pharynx, the epithelial cells of the oesophagus form an irregular pseudostratified cuboidal epithelium with cilia lining the internal surface (Fig. 3.5I). In the epithelium of the oesophagus there is a minority cell type (highlighted by the arrowhead) that has a weakly stained nucleus and prominent nucleoli. There are also a number of cells with mucus filled vacuoles (mu). As the oesophagus approaches the stomach the epithelium becomes more heavily stratified and contains more mucus-secreting cells and cilia. Surrounding the epithelium of the oesophagus there is a small amount of connective tissue (ct) and a thin layer of circular smooth muscle (ms).

Stomach. The cells of the stomach (Fig. 3.5J) resemble those lining the oesophagus but the epithelium of the stomach is more folded than the oesophagus and contains

gastric pits (gp). Like the oesophagus the stomach contains the cell type with the lightly stained nucleus (arrowhead) and many cells with mucus filled vacuoles. The epithelium of the stomach is surrounded by a thin layer of circular smooth muscle with almost no connective tissue present (Fig. 3.5J).

After the body of the stomach there is a short region of the gut called the transitional zone. The epithelium of the transitional zone has a folded epithelium containing ciliated (ci) and mucus secreting cells (mu) (Fig. 3.5K). This epithelium of the transitional zone is similar to that of the stomach so this region is considered here as an extended part of the stomach (discussed in chapter 3.5). The transitional zone like the rest of the stomach has a single layer of smooth muscle and very little connective tissue.

Small intestine. Part way along the region of the gut labelled sia there is a gradual change from the epithelium of the transitional zone to that of the absorptive small intestine. This absorptive intestinal epithelium consists of very tall columnar cells with their nuclei positioned in the basal half of the cell (Fig. 3.5L). It is towards the beginning of this tall epithelium that the common bile duct enters the intestine. The luminal side of the small intestinal epithelium has a well defined brush border (bb). This absorptive type epithelium constitutes the vast majority of the small intestine (sia-sic4). The small intestine, like the oesophagus and stomach, has almost no connective tissue and only a thin layer of smooth muscle (Fig. 3.5K+L).

Large intestine. The epithelial cells of the large intestine also form a columnar epithelium (Fig. 3.5M). Although this is less tall than the epithelium of the small intestine (Fig. 3.5L) the change in height of the epithelium along the intestine is a gradual one. Therefore, at this magnification the boundary from small to large intestine can not be identified by changes in histology. The boundary between the small and large intestine is defined based on the large increase in diameter, seen at the start of the large intestine (Fig. 3.5E, arrowhead). The large intestine also has little connective tissue and a thin smooth muscle layer (Fig. 3.5M).

Proctodaeum. The most posterior section of the gut, the proctodaeum, consists of a simple epithelium of small and rounded cells (Fig. 3.5N). The change from the columnar epithelium of the large intestine to the epithelium of the proctodaeum occurs roughly at the position that the gut passes through the body wall. The proctodaeum is surrounded by loose packed mesenchyme (Fig. 3.5N).

Liver, gall bladder and pancreas. The cells of the organs associated with the digestive tract, the pancreas, liver and gall bladder, are also clearly differentiated after seven days of development. The cells of the liver are small, rounded and arranged into close packed groups (Fig. 3.5O). There is considerable space between these groups of cells. The gall bladder consists of small cells arranged in a dense cuboidal epithelium (Fig. 3.5P). At this stage the cells of the pancreas appear as closely packed acinar cells (Fig. 3.5Q). The endocrine cells do not form distinguishable islets until later in development (Nieuwkoop and Faber, 1967).

The histology described here is consistent with the limited previous studies on specific parts of the *Xenopus* gut. For example, it has been reported that except for the typhlosole (an infolding in the anterior intestine that develops after the stages described here) (Ishizuya-Oka and Shimozawa, 1987; Nieuwkoop and Faber, 1967) there is little connective tissue in the *Xenopus* intestine up to metamorphosis(Ishizuya-Oka and Shimozawa, 1987). The single layer of smooth muscle has also been previously reported with the longitudinal muscle layer developing around stage 50 (Kordylewski, 1983).

3.4 THE RESPIRATORY SYSTEM

The endoderm gives rise to the epithelium of the respiratory system as well as the epithelium of the gut. The tadpole respiratory system is comparatively simple consisting of the gills, trachea and lung buds. The gills (gi) are located in the lateral pharynx and have a rake like appearance (Fig. 3.4A, (Nieuwkoop and Faber, 1967)). The trachea (tr) bifurcates from the posterior pharynx and runs in a posterior direction just ventral to the oesophagus (Fig. 3.4B). After a short distance the trachea splits to give rise to two lung buds (lu, Fig. 3.4C) which run into the body cavity. The

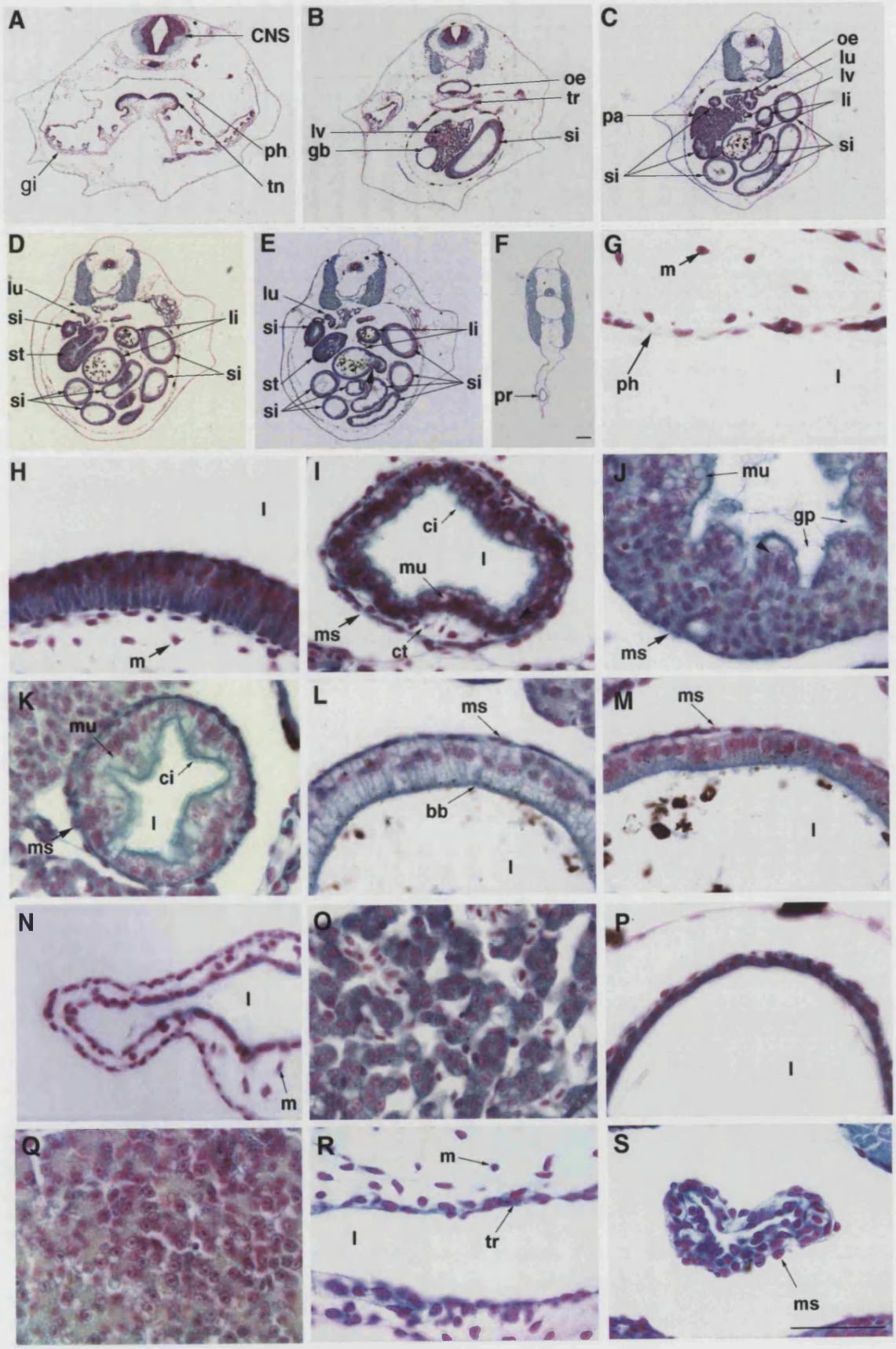
cells of the trachea form a squamous epithelium, which is surrounded by mesenchyme (Fig. 3.4R), while the epithelia of the lung is a stratified cuboidal epithelium with some mucus and cilia lining the apical side (Fig. 3.4S). Like the gut epithelium, the lung epithelium is surrounded by a single layer of smooth muscle (ms).

3.5 DISCUSSION

This study identifies all the organs of the gut and respiratory system and describes the anatomy and histology of these organs during development. The drawings of sections make up an atlas of *Xenopus* sections that allow the identification of any part of the gut found in a section. The position of a given region relative to the rest of the gut can then be easily established by reference to the schematic diagrams and wholemount drawings. The description of the histology enables these organs to be identified without reference to other landmarks in the embryo. This histology based identification could be particularly useful in identifying the organs in cases of abnormal development or in the case of cultured explants taken from early embryos. This study forms the foundation for the rest of the work in this thesis and hopefully other studies into the development of the endoderm.

If *Xenopus* tadpoles are going to be a good model for studying gut development then it is important that their gut is similar to the mammalian gut. At a gross level the tadpole gut has the same organs as the mammalian gut. At the cellular level the histology described here seems rather different from the familiar histology of the adult mammalian gut, but is in fact quite similar to the late embryonic or foetal gut (Morson *et al.*, 1990). For example, the mammalian foetal oesophagus is a ciliated stratified cuboidal epithelium before it undergoes metaplasia into a squamous epithelium (Morson *et al.*, 1990). The early intestine is lined with a simple columnar epithelium and the distal hindgut with a squamous epithelium.

Figure 3.5. Histology of the 7 day old *Xenopus* gut. All sections are stained with borax carmine (nuclear) and picroblue black (cytoplasmic). A-F, low power micrographs (scale bar 100 μm). G-S, high power micrographs (scale bar 50 μm). A, pharynx. B, liver and gall bladder. C, pancreas. D, stomach. E, border between small and large intestine (marked with arrow head). F, Proctodaeum. G, roof of pharynx. H, tongue. I, oesophagus (arrow head marks a minority cell type). J, stomach (arrow head marks a minority cell type). K, transitional zone. L, small intestine. M, large intestine. N, proctodaeum. O, liver. P, gall bladder. Q, pancreas. R, trachea. S, lung bud. Abbreviations, ph, pharynx, CNS, central nervous system, tn, tongue, lv, liver, gb, gall bladder, si, small intestine, li, large intestine, oe, oesophagus, tr, trachea, pa, pancreas, pr, proctodaeum, m, mesenchyme, ms, muscle, ct, connective tissue, l, gut lumen, mu, mucus, ci, cilia, gp, gastric pits, bb, brush border, lu, lungs, gi, gills, st, stomach.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The main difference between the tadpole and mammalian gut is that the mammalian intestine quickly develops a complex epithelium. This epithelium is also surrounded by a greater amount of connective tissue and two layers of smooth muscle. During metamorphosis, the *Xenopus* intestine develops more connective tissue, two layers of smooth muscle and a highly folded epithelium (Nieuwkoop and Faber, 1967). This has led to the suggestion that the development of the *Xenopus* intestine occurs in two phases (Shi and Ishizuya-Oka, 1996). Early development produces the simple intestine of the tadpole, which does not develop further until metamorphosis. At metamorphosis the intestine goes through a second phase of development which produces a more complex epithelium and smooth muscle layers, that is more similar to the mammalian gut.

Another difference between the *Xenopus* and the mammalian gut is that the *Xenopus* gut contains the transitional zone after the body of the stomach. This region has been previously described in the frog *Rana temporaria* (Barrington, 1946; Bodegas et al ., 1997) and in the toad *Bulfo bulfo* (Barrington, 1946) but is not present in the mammalian or chick gut. This region occurs after the body of the stomach, so has been previously classified as part of the intestine (Bodegas *et al.*, 1997). However, the cell types in the transitional zone are more similar to the oesophagus and stomach than the intestine, so it is considered here, and previously (Barrington, 1946), as an extended part of the stomach. The character of this region will be further discussed in chapter 4.

In general, the similarities between the mammalian and the tadpole gut support the idea that studies on the *Xenopus* gut will be relevant to the mammalian gut as well as to other amphibia.

CHAPTER 4 A DISSECTED GUT PREPARATION

4.1 INTRODUCTION

The preceding description of the tadpole gut was based on histological sections which allow an accurate picture of the three dimensional organisation and cell types of the tadpole gut. The disadvantage of working with sections is that they are time consuming to produce and it is difficult to look at gene expression in sections. To overcome these limitations a wholemount gut preparation was developed. The wholemount guts are quick to produce and allow all the organs of the gut (excluding the pharynx and oesophagus) to be easily viewed. The preparation also makes it possible to rapidly investigate gene expression in the gut using wholemount *in situ* hybridisation.

In this chapter the anatomy of 3, 5, and 7 day old dissected guts (stage 40/41, 45/46, 46/47) is described. The enzymatic activity of alkaline phosphatase and the gene expression of three *Xenopus* endodermal marker genes (*IFABP*, *endodermin* and *Xlhbox8*) is then examined. These expression patterns confirm the previous histology based identifications of the organs. Lastly the expression of three transcription factors (*Xsox2, Xcad1* and *Xcad2*) whose expression has not been previously described in the *Xenopus* gut is examined.

4.2 A DISSECTED PREPARATION FOR THE *XENOPUS* TADPOLE GUT

After 3 days of development the dissected gut appears as a fairly thick tube (Fig. 4.1A+B). The stomach is orientated dorsal ventrally on the left of the embryo. The proximal part of the stomach can be seen on the dorsal side (Fig. 4.1A) and the distal part of the stomach on the ventral side (Fig. 4.1B). The distal portion of the stomach merges with the intestine. The prospective liver is the most anterior structure of the dissection while the pancreas lies between the stomach and the intestine on the left of the embryo.

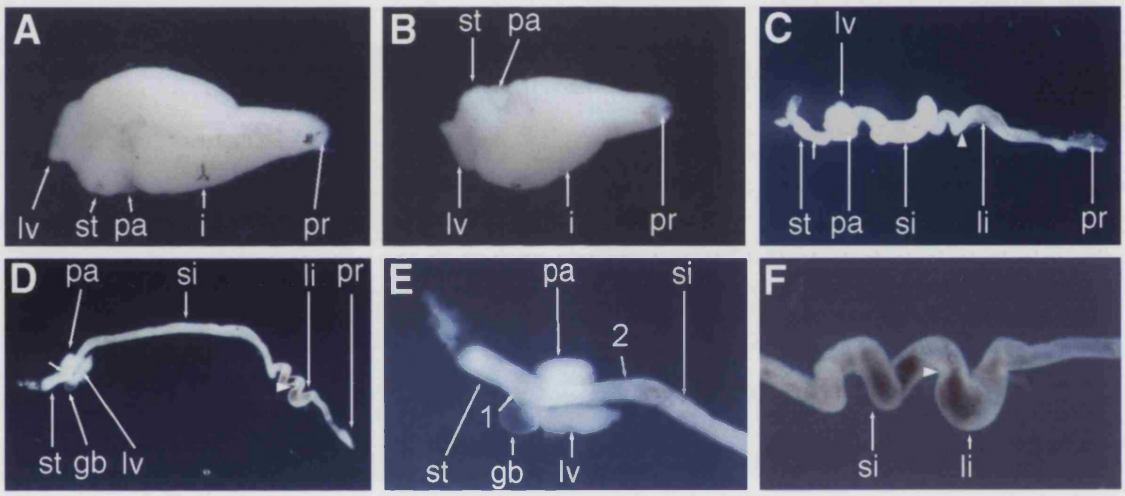
Two days later, the intestine has elongated considerably and it is possible to identify all of the organs of the gut (Fig. 4.1C). This elongation continues making the 7 day gut even longer and thinner (Fig. 4.1D-F). The following description is based on the 7 day gut but holds true for the 5 day gut. The main difference being the intestine is shorter and that the boundary between the small and large intestine is less obvious in 5 day old guts.

The stomach is the most proximal portion of the preparations (Fig. 4.1D+E). After the stomach there are two boundaries in the gut (Fig. 4.1E). The first boundary, labelled 1, marks the end of the stomach and the start of the transitional zone. After this boundary there is the short thin transitional zone and then a second boundary, marked 2. The second boundary marks the end of the transitional zone and the start of the absorptive small intestine. These boundaries are not identified by clear changes in anatomy so they are positioned based on boundaries of gene expression (see chapter 4.3+4.4) and histology (see chapter 3.3). The absorptive small intestine accounts for a large proportion of the length of the gut. Following the small intestine there is an increase in width that marks the boundary between the small and large intestine (li) (Fig. 4.1F, arrowhead). The large intestine gradually becomes thinner and merges with the final section of the gut, the proctodaeum (pr). This change is not marked by a clear anatomical boundary. The pancreas, liver and gall bladder can be clearly seen abutting the thin section of the small intestine (Fig. 4.1E). The gall bladder is attached to the anterior section of the liver making it easy to distinguish between the liver and pancreas.

4.3 EXPRESION OF ENDODERMAL MARKERS IN THE TADPOLE GUT

Having described the visible anatomy the activity of alkaline phosphatase and the expression of three commonly used endodermal marker genes was characterised in the dissected guts.

Figure 4.1. Anatomy of dissected *Xenopus* guts. A, dorsal view of a gut from a 3 day old embryo. B, ventral view of a gut from a 3 day old embryo. C, gut from a 5 day old embryo. D, gut from a 7 day old embryo. Arrow marks the boundary between the stomach and intestine. Arrowhead marks the boundary between small and large intestine. E, stomach region from a 7 day old gut. The first boundary (1) is between the stomach and the transitional zone and the second boundary (2) is between the transitional zone and the absorptive intestine. F, boundary between small and large intestine of 7 day old gut. The arrowhead marks the boundary between the small intestine and the large intestine. st, stomach, i, intestine, si, small intestine, li, large intestine, pr, proctodaeum, pa, pancreas, lv, liver, and gb, gall bladder.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li
pr
st
gb
lv
E
pa
si
2
1
st
gb
lv
F
si
li

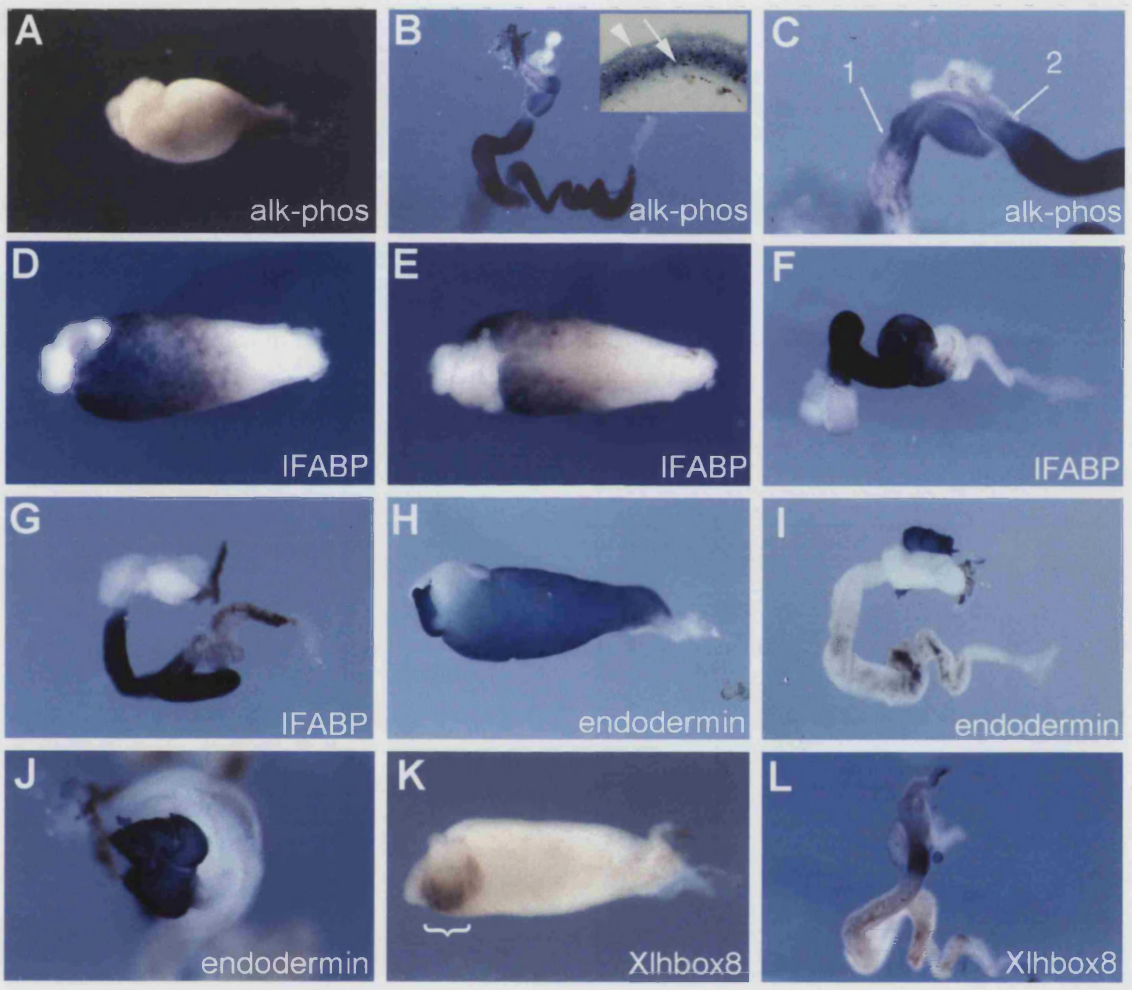
Alkaline phosphatase. In order to identify the boundary between the stomach and intestine the activity of endogenous alkaline phosphatase was examined. At 3 days of development alkaline phosphatase activity is only present in a small part of the posterior intestine (Fig. 4.2A). This small amount of activity is probably due to the lack of tissue differentiation. At 7 days of development there is alkaline phosphatase activity from the start of the small intestine to almost the end of the gut (Fig. 4.2B). At the proximal end of a 7 day old gut there is a sharp boundary (marked 1) in alkaline phosphatase activity marking the end of the stomach and the start of the transitional zone (Fig 4.2C). Through the transitional zone, the thin section of gut following the body of the stomach, there is low level expression of alkaline phosphatase. As the width of the gut increases there is a second boundary (marked 2) in alkaline phosphatase activity between the low levels in the thin section and the much higher levels of the small intestine. At the other end of the gut, the distal region which does not express alkaline phosphatase probably corresponds to the proctodaeum (Fig. 4.2B). Alkaline phosphatase activity was also present in the pancreas but not the liver.

IFABP is a thyroid hormone regulated gene identified as an intestinal fatty acid binding protein based on its high sequence similarity with mammalian *IFABP* (Shi and Hayes, 1994). In 3 day dissected guts *IFABP* expression was found throughout a large part of the prospective intestine (Fig. 4.2D+E). Interestingly there was less expression dorsally than ventrally (compare Fig. 4.2D to 4.2E). No expression was seen in the stomach, liver or pancreas. After 5 and 7 days of development *IFABP* expression can be seen to be restricted to the small intestine (Fig. 4.2F+G). The anterior boundary of *IFABP* expression was positioned just after the transitional zone (Fig. 4.2G) at the start of the absorptive intestine. This matches the second boundary in alkaline phosphatase expression. *IFABP* expression does not extend throughout all of the small intestine but stops before the boundary with the large intestine. The expression of *IFABP* seen in the dissected guts matches that seen for gene (Shi and Hayes, 1994) and protein expression (Ishizuya-Oka *et al.*, 1997) previously reported in sections.

Endodermin is a protease inhibitor that is expressed throughout the endoderm during early development (Sasai *et al.*, 1996). To date the expression pattern of *endodermin* has not been reported after stage 36 (late tail bud). After 3 days of development (stage 40/41) *endodermin* is still expressed throughout the *Xenopus* gut (Fig. 4.2H). However, at this stage there appears to be lower expression in the stomach and pancreas than in the rest. After 5 or 7 days of development *endodermin* is only expressed at a high level in the liver (Fig. 4.2I+J). Some dissected guts did show very low levels of expression in the intestine. However, this expression is much lower than the expression in the liver and the expression seen throughout the 3 day old gut. Therefore, the expression of *endodermin* changes from being pan-endodermal to being specifically expressed in the liver. This raises the possibility that *endodermin*, apart from being a useful pan-endodermal marker at early stages may have a specific role in liver development.

XlHbox8 is a homeodomain containing transcription factor (Wright *et al.*, 1988) and the homologue of *IPF-1* which is required for mammalian pancreas development (Jonsson *et al.*, 1994). At 3 days of development *XlHbox8* is expressed in the pancreas and in a narrow band of the gut near where the stomach meets the intestine (Fig. 4.2K, marked with a bracket). At 5 days of development *XlHbox8* is expressed in a small portion of the small intestine just after the transitional zone (Fig. 4.2L). There is also some very weak staining in the pancreas. The gall bladder is stained in this preparation but this occurred with all probes that required a long reaction time and is probably caused by trapping of probe or antibody. The expression pattern of *XlHbox8* in the dissected guts corresponds well with the reported protein expression pattern (Wright *et al.*, 1988). In sections of the late pancreas the Xlhbox8 protein is only expressed in about 10 % of the epithelial cells which is probably why the expression in the pancreas of later guts appears very weak.

Figure 4.2. Expression of endodermal markers in dissected *Xenopus* guts. A, activity of endogenous alkaline phosphatase in 3 day gut. B, activity of endogenous alkaline phosphatase in 7 day gut. Insert shows a section through stained gut (arrow highlights alkaline phosphatase staining (blue) in epithelium, arrow head highlights mesodermal tissue counterstained green). C, activity of endogenous alkaline phosphatase in stomach region of 7 day gut (1+2 show the 1st and 2 nd expression boundaries). D, Ventral view of *IFABP* expression in 3 day gut. E, dorsal view of *IFABP* expression in 3 day gut. F, *IFABP* expression in 5 day gut. G, *IFABP* expression in 7 day gut. H, *endodermin* expression in 3 day gut. I, *endodermin* expression in 5 day gut. J, *endodermin* expression in 5 day liver. K, left view of *XlHbox8* expression in 3 day gut (highlighted by bracket). L, *XlHbox8* expression in 7 day gut.


A
alk-phos
B
alk-phos
C
1
2
alk-phos
D
IFABP
E
IFABP
F
IFABP
G
IFABP
H
endodermin
I
endodermin
J
endodermin
K
Xlhbox8
L
Xlhbox8

The disadvantage of working with wholemount guts is that without sectioning the gut it is not possible to tell whether the expression is in the epithelium or the smooth muscle. Therefore, the expression described here could be in the smooth muscle or in the epithelium of the gut. *IFABP, endodermin* and *Xlhbox8* have all been previously shown to be expressed in the epithelia and sectioning of the wholemount guts confirmed this. The activity of alkaline phosphatase was also found to be in the epithelial layer (Fig. 4.2B insert).

4.4 EXPRESSION OF REGION SPECIFIC TRANSCRIPTION FACTORS IN THE TADPOLE GUT

IFABP, endodermin and *Xlhbox8* are the most commonly used markers of the late *Xenopus* endoderm. However, more genes with region specific expression patterns will need to be identified before the molecular mechanisms of gut formation can be established. It is particularly important to identify genes such as transcription factors and signalling molecules that, as well as being useful regional markers, could play a role in gut formation. In other vertebrates several transcription factors have been shown to have region specific expression patterns in the gut. The expression of the *Xenopus* homologues of three of these genes was examined in the tadpole gut.

Xsox2. The HMG box containing transcription factor *Sox2* has been shown to be expressed in the anterior chick endoderm (Ishii *et al.*, 1997). The distal boundary of *sox2* expression marks the distal end of the stomach making this gene a potentially useful marker of the anterior endoderm.

In the 3 day gut the *Xenopus* homologue of *sox2, Xsox2* (Mizuseki *et al.*, 1998), was found to be expressed in the presumptive oesophagus and stomach (Fig. 4.3A). The distal boundary of this anterior domain matches the position where the expression of *IFABP* starts (Fig. 4.2D). It seems likely that the border between *Xsox2* and *IFABP* expression marks the end of the presumptive stomach and the start of the presumptive small intestine. Interestingly *Xsox2* also had a posterior domain of expression in the 3 day gut (Fig. 4.2A). This expression is right at the distal end of

the gut and could correspond to the future proctodaeum. The pancreas and liver of the 3 day gut did not express *Xsox2*.

In the 7 day gut, as in the 3 day gut, *Xsox2* had a proximal region of expression (Fig. 4.3B+C). This expression domain extended right through the stomach to the end of the transitional zone (Fig. 4.3C). However, similar to the activity of alkaline phosphatase, this domain contained two regions with different levels of expression. The proximal, lower expressing region, extends to the end of the body of the stomach (boundary marked 1, Fig. 4.3C). The second domain extends to the end of the transitional zone (boundary marked 2, Fig. 4.3C). The 3 day *Xenopus* gut had a distal as well as proximal *Xsox2* expression domain. In the posterior of the 7 day guts there is a very faint expression of *Xsox2* (Fig. 4.3D). This expression is very weak so expression in the distal domain seems to have been down regulated in these later guts. In the 7 day gut *Xsox2* is also expressed in the pancreas but not the liver (Fig. 4.3B+C). This new expression domain in the pancreas and the down regulation of the distal region of *Xsox2* expression shows that *Xsox2* expression is quite dynamic in the *Xenopus* gut.

The proximal expression domain of *Xsox2* that extends to the start of the intestine is consistent with that described in the chick (Ishii *et al.*, 1997). However, the distal and pancreas expression domains have not been reported in the chick. This could be because of species differences or it could be that these domains are present in the chick but have not been reported.

Xcad1 and Xcad2. The *caudal* genes are homeobox containing transcription factors that are related to the founding member of the family, the *Drosophila* gene *caudal* (Mlodzik *et al.*, 1985). *Caudal* genes have been isolated from many vertebrates with most species having 3 members of the family (discussed in Marom *et al.*, 1997). In *Xenopus* the three members are called *Xcad1, Xcad2* and *Xcad3* (Blumberg *et al.*, 1991; Northrop and Kimelman, 1994). As members of the *caudal* family from other vertebrates have been shown to be expressed in and play role in the development of the intestine (Chawengsaksophak *et al.*, 1997; Doll and Niessing, 1993; Suh *et al.*,

1994) the expression of the *Xenopus caudal* genes was characterised in the tadpole gut. *Xcad1* and *Xcad2* were found to be expressed in identical patterns so will be discussed together below. Expression of the third member of the *Xenopus caudal* family *Xcad3* was not detected in the tadpole gut.

In the 3 day gut *Xcad1+2* were found to be expressed throughout the presumptive intestine (Fig 4.3E+I). The proximal boundary of expression matched the distal boundary of *Xsox2* expression and the beginning of *IFABP* expression at the start of the small intestine. Distally expression extended through out the gut except for a small posterior region. This is in contrast to the expression of *IFABP* which is only expressed in the proximal intestine (Fig. 4.2D+E). The distal region of the gut that does not express *Xcad1+2* corresponds to the distal domain of *Xsox2* expression. Therefore in the 3 day gut the proximal and distal limits of *Xcad1+2* expression are flanked by *Xsox2* expressing regions.

Xcad1+2 were also expressed throughout the intestine in the 7 day gut (Fig 4.3 F,G, H and J, K ,L). As in the 3 day gut, the anterior boundary (Fig 4.3G+K) matched the distal boundary of *Xsox2* expression and the proximal boundary of *IFABP* expression. The distal expression domain, as in the 3 day gut, extended to almost the extreme distal end of the gut (arrow, Fig. 4.3 H+L). The very posterior domain that does not express *Xcad 1+2* probably corresponds to the proctodaeum.

The expression of these genes, as discussed above, could be in the smooth muscle or the epithelium of the wholemount guts. Sections of the wholemount guts showed that *Xsox2*, *Xcad1* and *Xcad2* were expressed in the epithelium of the gut. However, because the smooth muscle layer is so thin and in such close proximity to the epithelia it was not possible to be completely sure that these genes were not also expressed in the smooth muscle layer. In the future it will be important to establish if these genes are expressed in the smooth muscle (see chapter 8.3).

In summary, *Xcad1+2* are expressed throughout the intestine of the 3 and 7 day old gut. In the 3 day old guts the expression of *Xcad1+2* is flanked on both sides by

expression of *Xsox2*. It seems likely that the boundaries between *Xsox2* and *Xcad1+2* expression marks the future proximal and distal boundaries of the intestine. In the 7 day old gut the proximal boundary of *Xcad1+2* is still flanked by the expression of *Xsox2* but the distal boundary is no longer flanked by *Xsox2* because the distal domain of *Xsox2* expression has been down regulated. *Xsox2* is also expressed in the pancreas of the 7 day gut.

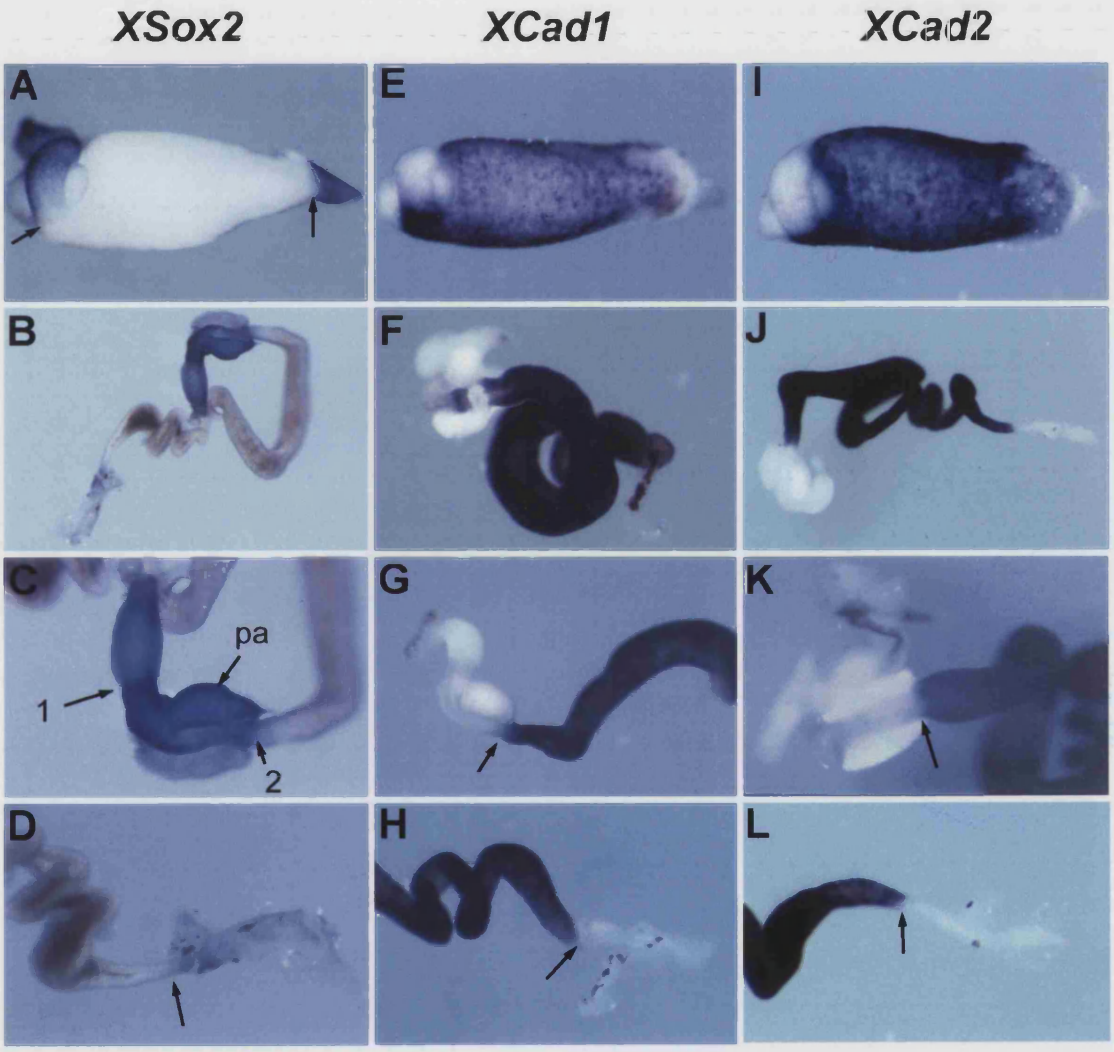
4.5 DISCUSSION

In chapter 3 the organs of the gut were identified based on their anatomy and histology. In this chapter a dissected gut preparation has been developed and the expression of 3 endodermal marker genes plus the activity of alkaline phosphatase described in the dissected guts. The expression patterns of the marker genes and the enzymatic activity of alkaline phosphatase described in this chapter confirm the previous histological based identifications. This shows that the description of gut development, that forms the basis for the rest of this study, is accurate and reliable.

The transitional zone lies between the body of the stomach and the small intestine and has previously been considered as both part of the stomach and part of the intestine (see chapter 3.5). Based on the histology of this region it was designated as an extended part of the stomach. Alkaline phosphatase activity was found in the transitional zone suggesting that it is intestinal in nature. However, the level of alkaline phosphatase activity was much lower than the true absorptive intestine and the other intestinal markers *IFABP* and *Xcad1+2* were not expressed in the transitional zone. *Xsox2* which is expressed in the stomach but not the absorptive intestine was also expressed in the transitional zone suggesting that it is gastric in nature. Therefore, based on the cell types and gene expression of the transitional zone it is considered here as an extended part of the stomach.

The expression patterns of *Xsox2* and *Xcad1+2* had not been previously described in the *Xenopus* gut. In chick embryos *Xsox2* is expressed in the anterior endoderm and it was hoped that it would be a useful marker of the anterior endoderm. In *Xenopus*

Figure 4.3 Expression of region specific transcription factors in dissected *Xenopus* guts. A, *Xsox2* expression in the three day gut. The proximal and distal boundaries of expression are shown with arrows. B, *Xsox2* expression in the 7 day gut. C, proximal expression of *Xsox2* in the 7 day gut. The two boundaries in expression are marked (1 and 2). Pa, pancreas. D, very faint expression of *Xsox2* in the distal 7 day gut. E, *Xcad1* expression in the three day gut. F, *Xcad1* expression in the 7 day gut. G, proximal expression of *Xcad1* in the 7 day gut. Arrow shows proximal boundary of expression. H, expression of *Xcad1* in the distal 7 day gut. Arrow shows distal boundary of expression. I, *Xcad1* expression in the three day gut. J, *Xcad1* expression in the 7 day gut. K, proximal expression of *Xcad1* in the 7 day gut. Arrow shows proximal boundary of expression. L, expression of *Xcad1* in the distal 7 day gut. Arrow shows distal boundary of expression.


XSox2
XCad1
XCad2
A
E
I
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C
pa
1
2
G
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L

Xsox2 was found to have domains of expression in the proximal and distal gut and in the pancreas. These extra domains mean that it may not be so useful as an anterior marker. However, its expression seems to flank both sides of the intestine so it could be valuable for investigations into how the boundaries of the intestine form. *Xcad1+2,* like other vertebrate *caudal* genes, were found to be expressed in the intestine, so they should be useful markers for this tissue. This is particularly true of the distal small intestine and large intestine as the currently used intestinal marker *IFABP* is not expressed in these regions. *Xsox2*, *Xcad1+2* and the previously described endodermal markers represent a useful panel of marker genes for the study of endoderm development. They could be used to identify tissue in specification experiments or for scoring the effect of experimental manipulations on the endoderm.

Xlhbox8, Xsox2 and *Xcad1+2* are also likely to play a role in encoding the regional identity of the gut. There is already evidence from other vertebrates that the mammalian homologue of *Xlhbox8* is involved in pancreas development (Jonsson *et al.*, 1994) and that the *caudal* genes are involved in intestinal development (eg Chawengsaksophak *et al.*, 1997; Suh *et al.*, 1994). This data makes it very likely that *Xlhbox8* and *Xcad1+2* are important in the development of the organs of the tadpole gut. To date there is no evidence that *sox2* plays a role in gut development but its dynamic expression pattern in the gut make it very likely that it has several roles in gut development.

A final point regarding these molecular markers is that their expression in the tadpole gut is very similar to that of their homologues in the gut of other vertebrates, showing that their expression has been conserved during evolution. This is important for two reasons: First, it means that looking at the expression of homologues of genes expressed in the gut of other species is likely to continue to be a good way of finding interesting genes expressed in the *Xenopus* gut. Secondly it suggests that the mechanisms of gut development in *Xenopus* are likely to be similar to those operating in other vertebrates. This is further evidence that *Xenopus* is likely to be a good model organism for the study of gut development.

In future investigations this simple method of visualising the gut as a wholemount offers the potential for rapidly scoring the effect of experimental manipulations on the anatomy of the gut and on the expression of the molecular markers described here. In this study these gut preparations are used in the cell rearrangement experiments carried out in chapter 8.

CHAPTER 5 THE ENDODERM FATE MAP

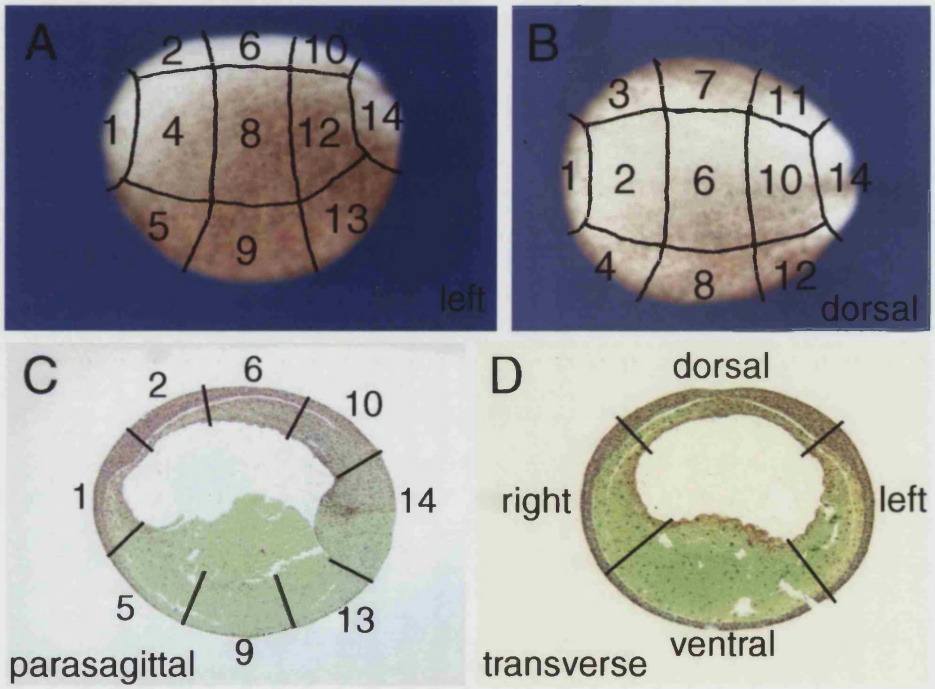
5.1 INTRODUCTION

In order to investigate the specification of the endoderm an accurate fate map is required so that the results of any explant or transplantation experiment can be compared with the presumptive fate for that piece of tissue. There are a number of existing amphibian endoderm fate maps (particularly Tahara and Nakamura, 1961) but these suffer from the limitations of spreading and fading associated with vital dyes and none of these older studies were carried out using *Xenopus* embryos. The currently available fluorescent dyes (Grimlich and Braun, 1985) and the preceding study of the tadpole gut mean that it is now possible to produce a comprehensive and accurate fate map for the endoderm. Grafts of fluorescent-labelled tissue were used to produce a new fate map showing which parts of the early endoderm give rise to which organs in the gut and respiratory system.

5.2 THE 14 EMBRYONIC REGIONS USED FOR THE FATE MAPPING

The early neurula stage *Xenopus* embryo was divided up into 14 regions that covered the entire embryo (Fig. 5.1). The 14 regions consisted of 1 at the very anterior, called "extreme anterior". Posterior to the extreme anterior region the embryo was split into 3 anterior/posterior levels, termed "anterior", "middle" and "posterior". Each of these levels was split into a dorsal, right, left and ventral region. Finally the most posterior region, the "extreme posterior" region, was opposite the extreme anterior region. Each of these regions was labelled using orthotopic grafts of fluorescently labelled tissue and the embryos left to develop to stage 46. They were then fixed and sectioned (see materials and methods) and the labelled epithelia scored. Examples of the experiments are shown in Figure 5.2. A photograph of the FDA label (FDA highlighted by arrow heads) and a labelled drawing of a section is shown for each

Figure 5.1. The 14 regions used for fate mapping. A, left view of a stage 14 wholemount. B, dorsal view of a stage 14 wholemount. C, parasagittal section of a stage 14 embryo. D, transverse section of a stage 14 embryo. The grafting regions have been shown on the embryos using the following labels; 1, extreme anterior, 2, anterior dorsal, 3, anterior right, 4, anterior left, 5, anterior ventral, 6, middle dorsal, 7, middle right, 8, middle left, 9, middle ventral, 10, posterior dorsal, 11, posterior right, 12, posterior left, 13, posterior ventral and 14 extreme posterior.


A
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6
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1
4
8
12
14
5
9
13
left
B
3
7
11
1
2
6
10
14
4
8
12
dorsal
C
2
6
10
1
14
5
13
9
parasagittal
D
dorsal
right
left
ventral
transverse

example. The drawing of the section makes it possible to easily identify the labelled organs. A wholemount drawing is also included to show where the sections originated from in the tadpole.

5.3 PRESENTING THE ENDODERM FATE MAP

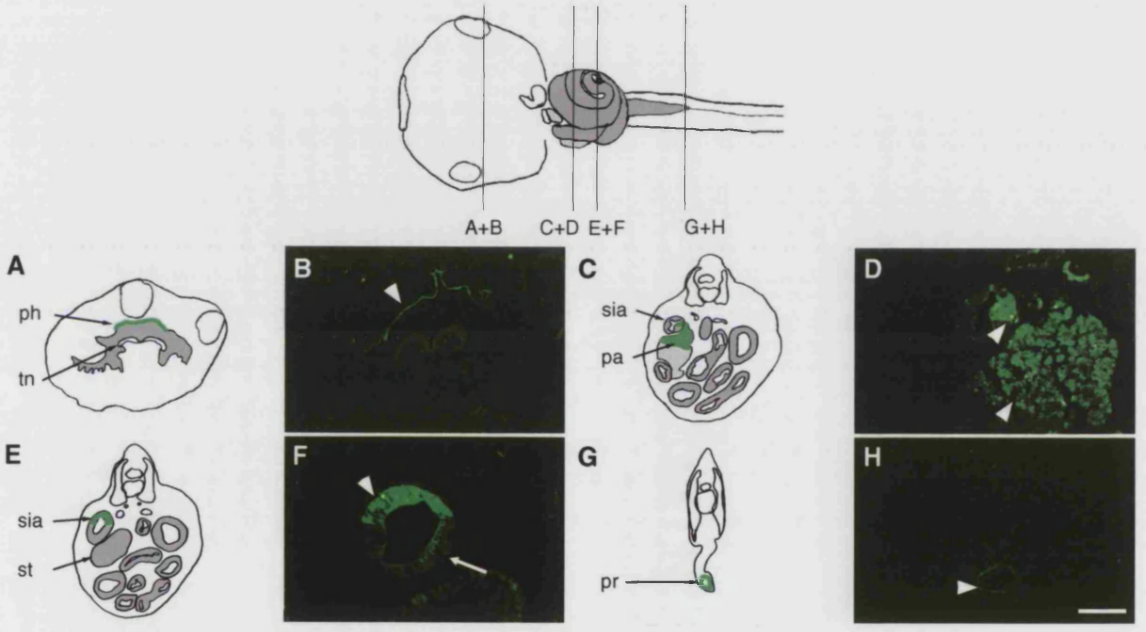
The results for the endoderm fate mapping are presented in three ways. Table 5.1 shows the organs that were labelled from grafts in each region. If a particular region labelled a particular organ in at least 50 % of cases then that organ was considered to be fated to form from that region and was included in the fate map (shaded grey in table 5.1). A limitation of this method is that it gives the same score if a graft labels a small or large proportion of cells of an organ. To overcome this the labelling pattern for a typical example for eight of the 14 regions is shown (Fig. 5.3). The six lateral regions label a similar proximal/distal part of the gut to the ventral regions so were not included here. Each of the 8 typical examples have drawings of sections from that embryo at a number of standard positions. The drawings are shaded green where the FDA label was present. A set of standard sections is shown above the typical examples, so that the labelled organs can be easily identified by comparison with these standard sections. The position of the section in the tadpole body can then be established by reference to the drawing of the wholemount. These diagrams give an indication of the amount of labelling in each organ. Finally we present the data in two types of summary diagrams (Fig. 5.4A-C). The first shows a drawing of a neurula stage embryo labelled with the organs that the extreme anterior, dorsal, ventral and extreme posterior regions are fated to form (Fig. 5.4A). For the sake of clarity the lateral regions, which gave similar results to the ventral regions, have not been included in the diagram (They are included in Figure 6.2A which will be discussed later as will 5.4D). The second type of diagram shows how the early endoderm projects on to the later gut (Fig. 5.4B+C). The projection of the ventral endoderm (with extreme anterior and extreme posterior regions) and the dorsal endoderm (with extreme anterior and posterior regions) is shown (Fig. 5.4B+C). It is

Table 5.1. The endoderm fate map

position	n	A ph	P ph	tn	gills	tr	lungs	liver	gb	pa	bd	oe	st	sia	sib	sic	lic	lid	pr
extreme anterior (1)	9	89	89	78	33	44	44	11		22	22	22	22	22	11				
anterior dorsal (2)	8	25	100	13						38		75	63	63					
anterior right (3)	6		50	17	33					67	50	83	100	83					
anterior left (4)	6		33	17	17	17	17			50		33	83	83					
anterior ventral (5)	9		67	67	44	55	55	100	67	89	89	78	78	100	22				
middle dorsal (6)	6									100		17	34	100	17				
middle right (7)	6											17	17	100	83	67			
middle left (8)	7												14	86	100	43			
middle ventral (9)	8													75	100				
posterior dorsal (10)	6									50				50	83	83	33	17	
posterior right (11)	6														83	100	50	33	
posterior left (12)	10													10	90	100	40	30	
posterior ventral (13)	6														50	83	67	67	
extreme posterior(14)	6																50	100	100

The percentage of cases that each organ was labelled is shown for each of the 14 regions. Organs that were labelled in at least 50% of the cases were included in the fate map and shaded grey in this table. Abbreviations; n, number of cases, A ph, anterior pharynx, P ph, posterior pharynx, tn, tongue, tr, trachea, gb, gall bladder, pa, pancreas, bd, bile duct, oe, oesophagus, st, stomach, sia, proximal small intestine, sib, external coil of the small intestine, sic, internal coil of the small intestine, lic, internal coil of the large intestine, lid, distal large intestine, pr, proctodaeum.

Figure 5.2. Examples of FDA labelling in the endoderm-derived epithelia. For each example a labelled drawing of a section (A, C, E, G) and a photograph of the labelled section is shown (B, D, F, H). A wholemount drawing is also included at the top of the figure to show the position of the sections in the tadpole. A + B, labelled pharynx. C + D, labelled pancreas and small intestine. E + F, labelled small intestine. G + H, labelled proctodaeum. In the photographs of FDA label (B, D, F, H) arrow heads highlight labelled epithelia and arrows highlight smooth muscle. Scale bar: B, 200 μm, D, 95 μm F+H, 120 μm. Abbreviations; ph, pharynx, tn, tongue, sia, proximal small intestine, pa, pancreas, st, stomach, pr, proctodaeum.


A+B
C+D
E+F
G+H
A
ph
tn
B
C
sia
pa
D
E
sia
st
F
G
pr
H

important to realise that the shaded regions of the gut are meant to show that labelled cells were found in these regions rather than every cell in these regions was labelled.

5.4 OVERVIEW OF THE ENDODERM FATE MAP

Each region was found to be reproducibly fated to form part of the gut or respiratory system. This shows that all regions of the endoderm contribute to the tadpole gut or respiratory epithelia. Not surprisingly, the anterior endoderm was found to be fated to form proximal gut epithelia while the posterior endoderm formed distal epithelia. In fact there was a smooth projection without discontinuity from early to late stages in relation to all three anatomical axes: anterior/posterior, dorsal/ventral and left/right. There was some deformation in that the dorsal endoderm was generally found to be fated to form more anterior structures than the ventral endoderm (discussed in more detail below). So, although the dorsal endoderm remains opposite the ventral endoderm it ends up opposite ventral cells that originated from a more anterior position in the endoderm.

The later gut has a left/right asymmetry and there is currently a lot of interest in how this is established (e.g. Campione *et al.*, 1999, reviewed by Yost, 1998). The fate mapping showed no convincing difference between the fate of the left and right grafts, so production of the asymmetry must occur without large differences in cell fate or migration between the right and left sides of the embryo.

5.5 SPECIFIC REGIONS OF THE ENDODERM FATE MAP

Anterior regions. The extreme anterior region (1) labelled the epithelium of the pharynx and the tongue (orange in figure 5.4). All the anterior grafts (2-5, red in figure 5.4) labelled the epithelium of the pharynx, oesophagus, stomach and proximal small intestine (sia). However, the anterior ventral (5) and lateral grafts (3+4) labelled less pharynx and more distal parts of sia than the anterior dorsal (2) grafts (compare 5.4B with 5.4C). So, the dorsal endoderm is fated to form more anterior structures than the lateral and ventral endoderm. The anterior ventral grafts (5) also labelled the trachea, lungs, liver, gall bladder, pancreas and bile duct showing that a

large number of organs are fated to form from this small anterior ventral portion of the endoderm. The anterior left (3) and right (4) grafts also labelled the pancreas but not the liver. This shows that the ventral pancreatic rudiment but not the liver has a lateral as well as ventral component.

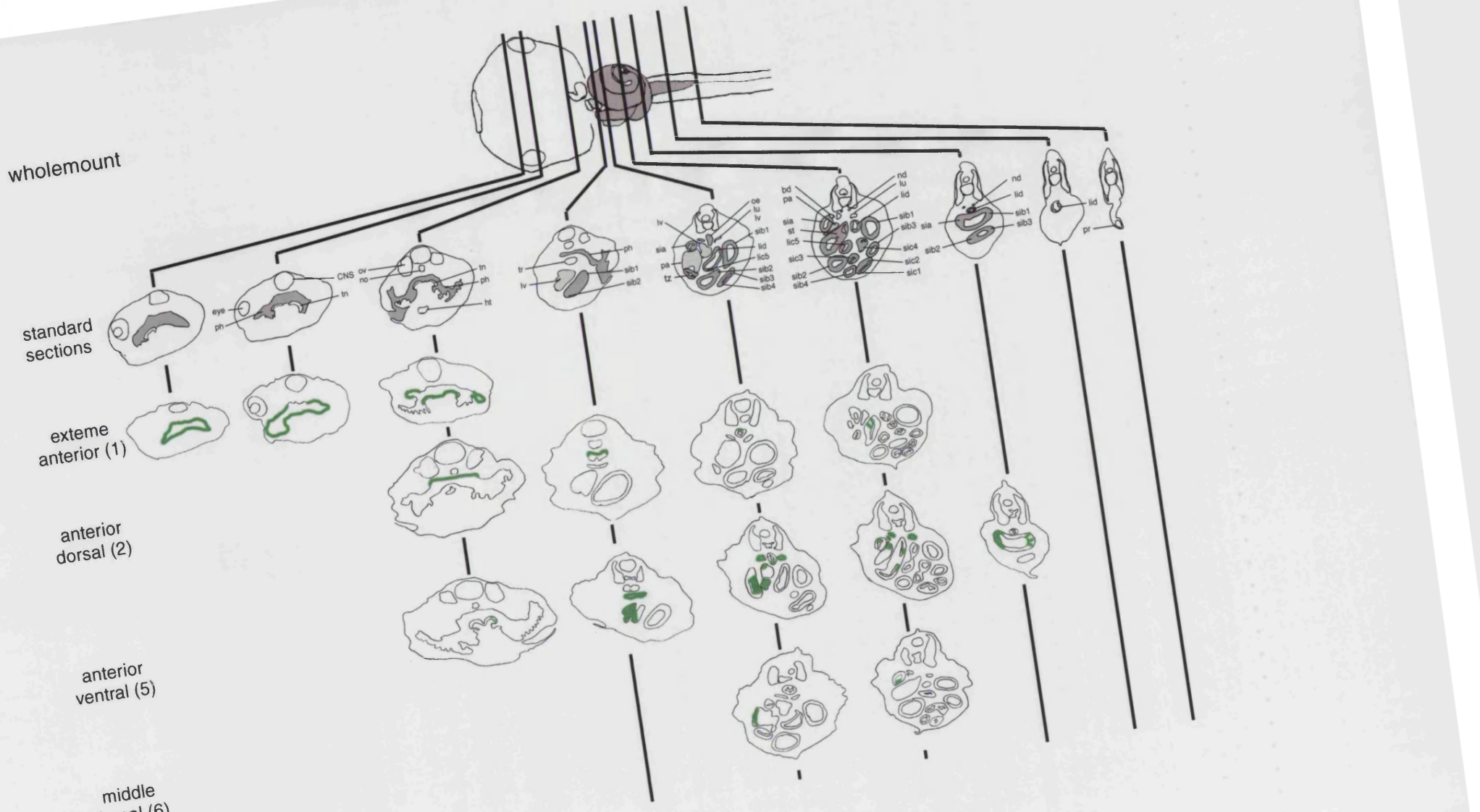
Middle regions. The middle dorsal region (6, blue in Figure 5.4) labelled the part of sia and the pancreas. This means that the dorsal pancreas forms from a more posterior region than the ventral pancreas. The middle right (7), left (8) and ventral (9) regions labelled the distal portion of sia and a large part of sib (blue in Figure 5.4). Therefore, like the anterior regions, the middle lateral and ventral grafts labelled more distal parts of the gut than the dorsal grafts.

Posterior regions. The posterior right (11), left (12), and ventral (13) regions (green in Figure 5.4) labelled the distal part of sib, sic and a small part of the large intestine. The posterior dorsal region (10) labelled cells in a large span of the intestine from sia through to sic. Therefore, the fact that the anterior and middle dorsal endoderm is shifted to the anterior compared with the lateral and ventral endoderm is compensated for by the spreading out of the posterior dorsal endoderm over a large part of the intestine (compare Fig. 5.4B+C). The extreme posterior graft (14, black in figure 5.4) labelled the most distal parts of the gut: the large intestine and the proctodaeum.

5.6 DISCUSSION

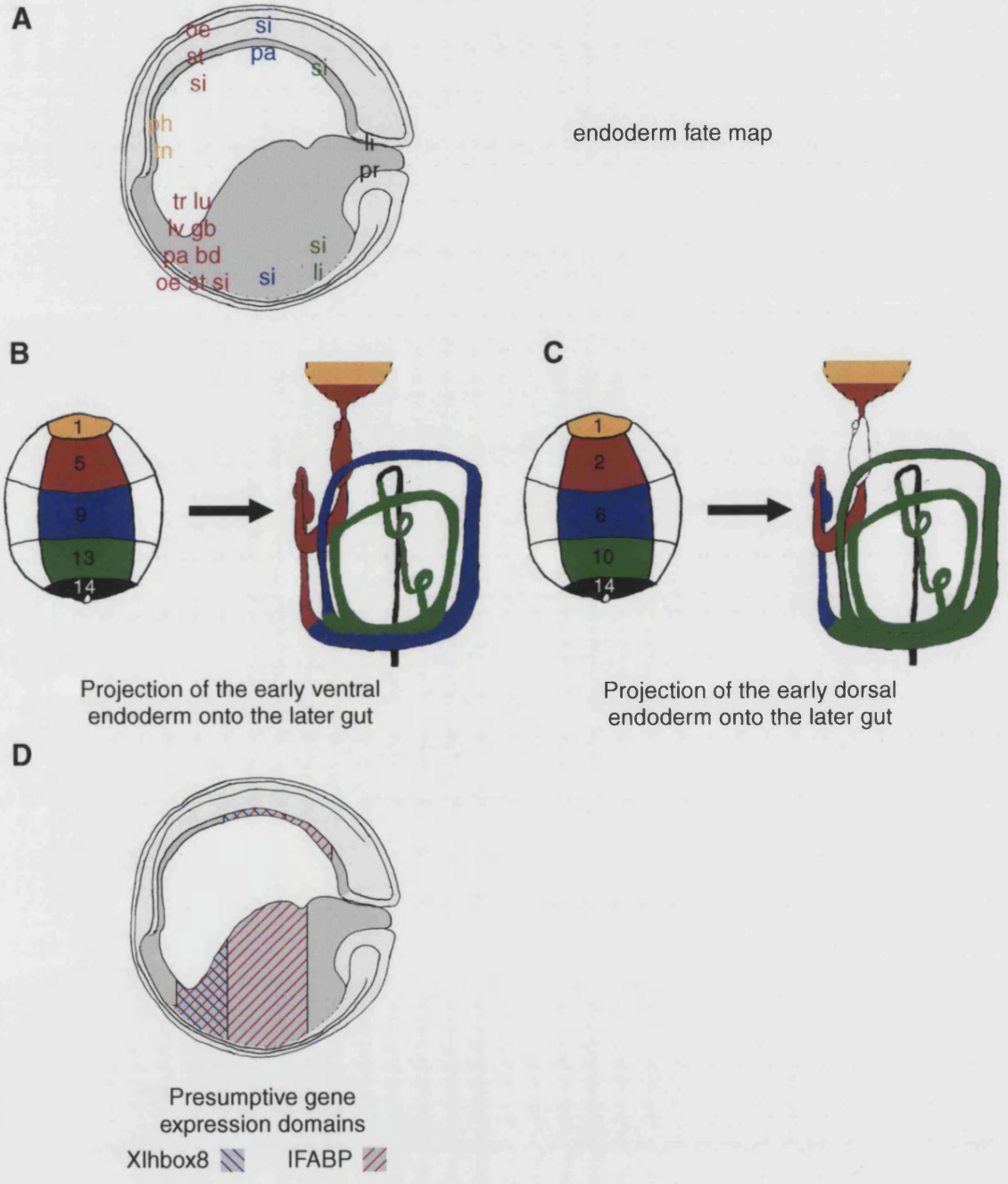
Extraembryonic endoderm in Xenopus. It has been proposed that an anterior region of the *Xenopus* endoderm and the extraembryonic endoderm in mammals have equivalent roles in head induction (Jones *et al.*, 1999). It has also been suggested, that as well as having similar signalling functions to the extraembryonic endoderm, some of *Xenopus* endoderm may actually be extraembryonic in nature (Beddington and Robertson, 1999).

Figure 5.3. Representative examples of the fate mapping. Drawings of sections from representative examples of 8 of the 14 regions are shown. The position of the FDA label has been added to the drawings (green). A wholemount drawing and drawings of standard sections are also shown to aid interpretation of the labelled sections. Abbreviations; ph, pharynx, tn, tongue, ov, otic vesicle, no, notochord, ht, heart, lv, liver, tr, trachea, st, stomach, sia, proximal small intestine, sib, external coil of small intestine, sic, internal coil of small intestine, lic, internal coil of large intestine, lid, distal large intestine, pa, pancreas, bd, bile duct, lu, lungs, nd, nephritic ducts, pr, proctodaeum, CNS, central nervous system.


wholemount
standard sections
exteme anterior (1)
anterior dorsal (2)
anterior ventral (5)
middle dorsal (6)
eye
ph
CNS
tn
ov
no
ht
tr
lv
sib1
sib2
oe
lu
sia
pa
tz
lid
lic5
sib3
sib4
bd
sic3
sic4
sic2
sic1
pr


middle
ventral (9)
posterior
dorsal (10)
posterior
ventral (13)
extreme
posterior (14)

Figure 5.4. The endoderm fate map. A, The endoderm fate map. The organ rudiments of the extreme anterior (1) (orange), anterior dorsal (2) and ventral (5) (red), middle dorsal (6) and ventral (9) (blue), posterior dorsal (10) and ventral (13) (green) and extreme posterior (14) (black) endoderm are labelled at the position they originate from on a drawing of a stage 14 embryo. For the sake of clarity the lateral rudiments have not been included. B, Projection of the early ventral endoderm onto the tadpole gut. The extreme anterior (1) (orange), anterior ventral (5) (red), middle ventral (9) (blue), posterior ventral (13) (green) and extreme posterior regions (14) (black) are shaded in the embryo and in the regions they will give rise to in the tadpole gut. The drawings are viewed from the ventral side with anterior at the top. C, Projection of the early dorsal endoderm. As in B but for the dorsal rather than ventral regions. D, Presumptive gene expression domains in the dorsal and ventral *Xenopus* endoderm. Regions of the endoderm that will give rise to tissues that express *Xlhbox8* or *IFABP* in normal development are highlighted with coloured diagonal lines. Abbreviations; ph, pharynx, tn, tongue, tr, trachea, lu, lungs, lv, liver, gb, gall bladder, pa, pancreas, bd, bile duct, oe, oesophagus, st, stomach, si, small intestine, li, large intestine, pr, proctodaeum.


A
oe
st
si
si
pa
si
ph
tn
li
pr
tr lu
lv gb
pa bd
oe st si
si
si
li
endoderm fate map
B
1
5
9
13
14
Projection of the early ventral endoderm onto the later gut
C
1
2
6
10
14
Projection of the early dorsal endoderm onto the later gut
D
Presumptive gene expression domains
Xlhbox8
IFABP

The endoderm fate map shows that all regions of the endoderm give rise to part of the gut or respiratory epithelia. This means that there is no large region of extraembryonic tissue in the *Xenopus* endoderm. However, these grafts did not specifically label certain parts of these regions, such as the floor of the archenteron, so it remained possible that parts of these regions could still be extraembryonic. The question of extraembryonic endoderm in *Xenopus* will be returned to in chapter 8.

Projection of the endoderm. The endoderm fate map also showed that the axes of the endoderm are maintained in the later gut. This means that the mechanisms of gut morphogenesis produce a smooth projection from the neurula to the tadpole stage. Although the axes of the endoderm were maintained the anterior and middle dorsal endoderm was found to form more proximal gut structures than the equivalent ventral endoderm. This anterior shift of the dorsal endoderm is compensated for by the most posterior dorsal endoderm spreading out to cover a large part of the gut. Convergent extension of the dorsal axial tissues continues to occur during neurula stages (reviewed in Keller, 1987) and could possibly account for this distortion of the dorsal endoderm. A similar anterior shift in the endoderm has also been shown to occur earlier in development in the *Cerberus* expressing dorsal leading edge of the gastrulating endomesoderm (Bouwmeester *et al.*, 1996). These cells move anteriorly and then ventrally to end up in the ventral foregut region. The distortion of the dorsal endoderm shown by the fate map could represent a continuation of this earlier movement.

In general, the presumptive organ rudiments are found fairly evenly spread across the embryonic endoderm but an exception to this is the anterior ventral region which has a large number of organ rudiments. This region is also of interest because it contains the *Cerberus* expressing cells that form the dorsal leading edge during gastrulation (see above). It will be important to understand how so many organs are induced to form from this anterior ventral region. For example, it would be interesting to know if this region contains precursor cells with more than one fate or closely spaced precursor cells with a single fate, two possibilities that this study does not distinguish between. The fate map also shows the difference in origin between the rudiments of

the dorsal and ventral pancreas. There is currently a lot of progress being made in pancreas development and it will be interesting to see how the mechanisms of pancreatic induction act at the different origins of the dorsal and ventral pancreas to produce a single organ (eg Hebrok *et al.*, 1998).

Comparisons with fate maps from other species. The anatomy of the early chick embryo is very different to the anatomy of early *Xenopus* embryos but despite these differences there are a number of similarities between the endoderm fate map presented here and the fate map for the chick endoderm (Matsushita, 1996b). The anterior/posterior organisation of the chick endoderm is maintained in the later gut and in the chick the dorsal endoderm also gives rise to more proximal epithelia than the lateral endoderm. A fate map showing where the endoderm ordinates from in the early Zebrafish embryo has recently been published (Warga and Nusslein-Volhard, 1999). This fate map is from a comparatively much earlier stage than our fate map, making only limited comparisons possible, but it also showed some regional restriction to the fields of cells that are fated to form different organs of the gut.

The endoderm fate map and gene expression domains. As discussed above, *IFABP* is expressed in the tadpole small intestine and *Xlhbox8* is expressed in the tadpole pancreas. The fate map shows which parts of the endoderm will form these regions of the gut making it possible to predict which regions of the endoderm will express these genes in later development (Fig. 5.4D). The remaining genes from this work and other genes that are shown to be expressed in the gut can be easily added to the fate map. This prediction of what genes a piece of tissue will express in normal development will be useful for specification studies.

The endoderm fate map and specification studies. The fate map shows which parts of the endoderm will form which organs of the gut (or express which genes) in normal development. It should now be possible to explant regions of the endoderm to see if, as well as being fated to form a particular organ, the region of interest has been specified to form that organ. This would show whether the endoderm has already been patterned at neurula stages or whether the patterning occurs later.

In summary, a comprehensive fate map has been produced which shows which parts of the endoderm are fated to form which organs of the gut or respiratory system. This shows that the early endoderm projects smoothly onto the later gut and that there are no large regions of extraembryonic endoderm. The fate map can now be used as a basis for specification studies to see to what extent pattern is present in the neurula stage endoderm.

CHAPTER 6 THE GUT SMOOTH MUSCLE FATE MAP

6.1 INTRODUCTION

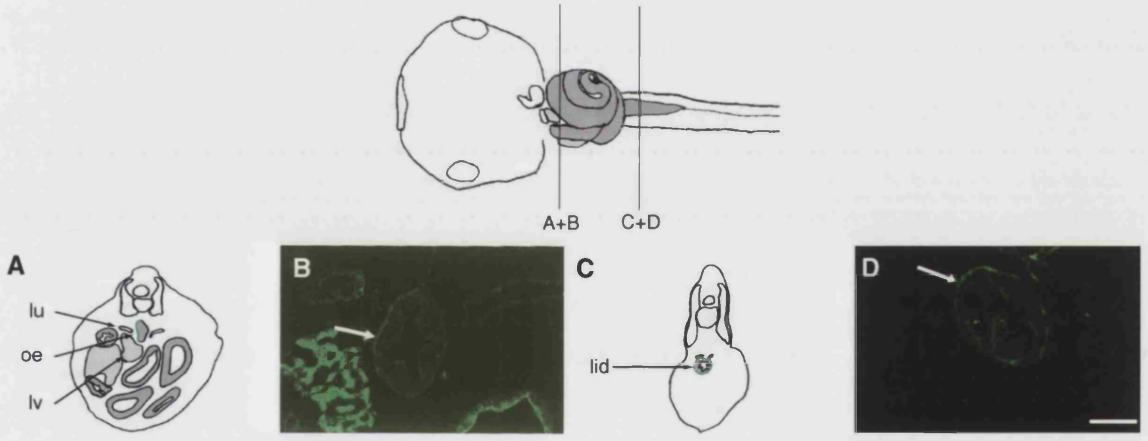
The smooth muscle that develops from the gut mesenchyme plays an important role in the development of the endoderm-derived epithelia (discussed in chapter 1.5). This means that in order to understand the development of the endoderm it is important to understand the origin of the smooth muscle. Prior to this study it was not known where the smooth muscle layer originates from in the *Xenopus* mesoderm, so a fate map was produced showing its origin.

6.2 THE FATE MAP FOR THE SMOOTH MUSCLE LAYER.

As the grafts used to construct the endoderm fate map also contained the mesoderm layer it was possible to score the label in the smooth muscle that surrounds the gut (Fig. 6.1, compare with the unlabelled smooth muscle in Fig. 5.2F).

The fate map for the smooth muscle layer is presented in table form (Table 6.1) and as a summary diagram (Fig 6.2B). Table 6.1 shows which organs' smooth muscle was labelled by grafts from each position. If grafts from a region labelled an organ in 50 percent or more of cases then that organ was included in the fate map for that position (shaded grey in Table 6.1). The summary diagram shows which organs of the gut the lateral and ventral mesoderm is fated to cover in smooth muscle (Fig. 6.2B). The lateral rudiments are shown in the middle of the dorsal/ventral axis to depict their origin from the side of the embryo. The mesoderm fate map can be compared with the endoderm fate map (Fig. 6.2A). For comparison with the smooth muscle fate map, this diagram only shows the organs of the digestive system that are covered in smooth muscle and unlike Figure 5.4A includes the lateral rudiments, shown in the middle of the embryo.

Figure 6.1. Examples of FDA labelling in the smooth muscle. For each example a labelled drawing of a section (A+C) and a photograph of the labelled section (B+D) is shown. A wholemount drawing is also included at the top of the figure to show the position of the sections in the tadpole. A+B, labelled smooth muscle in the oesophagus, C+D, labelled smooth muscle in the large intestine. Arrows highlight smooth muscle. Scale bar: B+D, 95 μm. Abbreviations; oe, oesophagus, lu, lungs, lv, liver, lid, distal large intestine.


A+B
C+D
A
lu
oe
lv
B
C
lid
D

The middle right (7) and left (8) grafts labelled the smooth muscle layer surrounding the oesophagus, stomach and sia. The middle ventral (9) grafts labelled the distal part of sia and sib. The posterior right (11) and left (12) grafts labelled the distal part of sib, sic and the proximal part of the large intestine. The posterior ventral (13) graft labelled the majority of the large intestine.

Table 6.1. Gut smooth muscle fate map.

position	n	oe	st	sia	sib	sic	lic	lid
extreme anterior (1)	9							
anterior dorsal (2)	8							
anterior right (3)	7(3)	29	14	29				
anterior left (4)	7(2)			43				
anterior ventral (5)	6							
middle dorsal (6)	6	16	16	16				
middle right (7)	4	50	75	100	25			
middle left (8)	5(1)	60	60	60	20			
middle ventral (9)	6			50	100			
posterior dorsal (10)	6							
posterior right (11)	6(4)				67	67	83	50
posterior left (12)	7(3)				43	100	43	14
posterior ventral (13)	7					29	57	100
extreme posterior(14)	6							

The percentage of cases that the smooth muscle for each organ was labelled is shown for each of the 14 regions. Organs that were labelled in at least 50% of the cases were included in the fate map and shaded grey in this table. The number of cases scored as wholemounts rather than in sections is shown in brackets. Abbreviations; oe, oesophagus, st, stomach, sia, proximal small intestine, sib, external coil of the small intestine, sic, internal coil of the small intestine, lic, internal coil of the large intestine, lid, distal large intestine, n, number of cases.

The other regions were not found reproducibly to contribute to the smooth muscle of the gut but were fated to form other mesodermal tissues, such as the notochord. Therefore, so the smooth muscle only originated from the middle and posterior lateral and ventral mesoderm. This fits well with a previous *Xenopus* fate map which

shows that the dorsal mesoderm forms axial tissues (eg notochord) while the anterior lateral mesoderm forms heart and head mesenchyme (Keller, 1976).

6.3 DISCUSSION

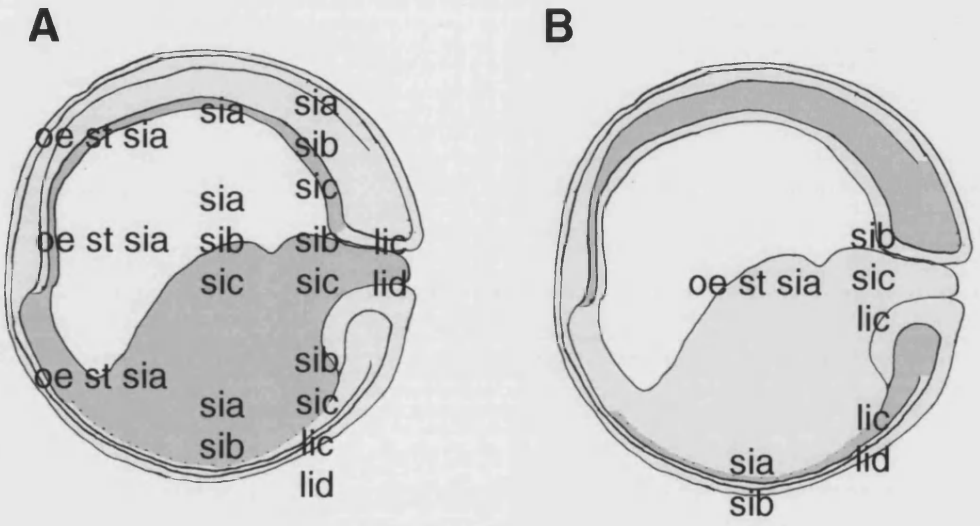
The smooth muscle fate map. The fate map shows where in the embryonic mesoderm the rudiments of the smooth muscle layer that covers the organs of the gut originates. Unlike the rudiments of the epithelia, the rudiments of the smooth muscle were restricted to the lateral and ventral mesoderm. The smooth muscle fate map, like the endoderm fate map, could now be used as a basis for explant experiments to see if the mesoderm from these regions is already specified to form smooth muscle.

Comparison with the chick smooth muscle fate map. There are a number of interesting similarities between this fate map and the fate map for the chick smooth muscle layer (Matsushita, 1995). The rudiments of the chick smooth muscle are also found in the lateral mesoderm but not in the midline. Another similarity is that in the chick the rudiments located next to the midline form more proximal smooth muscle than the rudiments located further laterally. This is the equivalent of the *Xenopus* lateral rudiments forming more proximal smooth muscle than the ventral rudiments. Therefore, despite the differences in anatomy between chick and *Xenopus* embryos, they show a number of similarities in the location of their epithelial and smooth muscle rudiments.

Origin of the smooth muscle layer and signalling between the mesodermal and endodermal tissues.

Comparison of the endoderm and smooth muscle fate maps shows that there were several differences between the origin of the epithelia and the smooth muscle. All regions in the endoderm were found to form part of the gut epithelium while the smooth muscle originated from a subset of regions in the early mesoderm. Another difference between the mesoderm and endoderm is that the lateral and ventral endoderm labelled the same proximal/distal segment of the gut. In contrast the lateral

Figure 6.2. The smooth muscle layer fate map. A, location of the dorsal, lateral and ventral endoderm digestive tract rudiments for comparison with the smooth muscle layer fate map. The lateral rudiments are shown in the middle of the dorsal/ventral axis to represent their position on the side of the embryo. B, smooth muscle layer fate map. The position in the mesoderm of the lateral and ventral rudiments for the smooth muscle layer of the gut is shown on a drawing of a stage 14 embryo. None of the dorsal regions were fated to form smooth muscle. Abbreviations; oe, oesophagus, st, stomach, sia, proximal small intestine, sib, external coil of the small intestine, sic, internal coil of small intestine, lic, internal coil of the large intestine, lid, distal large intestine.



Gut tube fate map
(endoderm)

Gut tube fate map
(mesoderm)

mesoderm is fated to form more proximal smooth muscle than the ventral mesoderm. These differences mean that for most organs of the gut the prospective smooth muscle does not overlay the corresponding prospective epithelia in the early embryo.

Comparison of the chick fate maps (Matsushita, 1995; Matsushita, 1996b) shows that the presumptive rudiments of the endoderm are located in a more anterior position than the corresponding ones in the mesoderm. This means that in both the chick and *Xenopus,* the two sets of rudiments are not in accord in the early embryo so they must move into accord at some point during development. It could be that the layers move into accord as the gut mesenchyme separates from the rest of mesoderm and surrounds the forming gut tube.

There is evidence that the early endoderm has some pattern before the gut tube forms (discussed in chapter 1.7). One possible explanation for this pattern was that the signals from the gut mesenchyme, demonstrated by the chick quail recombination experiments, start acting early in development before the mesenchyme separates from the rest of the mesoderm and surrounds the gut tube (discussed in chapter 1.5). However, the fate maps show that the two layers are not in accord in early development, which means that continuous inductive signals from the gut mesenchyme can not take place until later in development when they move into accord. This does not rule out signals from the early mesoderm to the endoderm but these signals must be different from the later signals that have been shown to originate from the gut mesenchyme. Therefore, comparison of the fate maps shows that any pattern in the early endoderm can not be caused by these previously demonstrated signals, so must be caused by an alternative and unknown mechanism.

In the light of the smooth muscle fate map, it is also interesting to look at the classic amphibian recombination experiments (discussed in chapter 1.4). These experiments show that the early mesoderm, rather than the gut mesenchyme which was used in the chick/quail experiments, is capable of respecification of the endoderm. Okada found that endoderm combined with anterior or dorsal mesoderm produced mainly anterior epithelia. In contrast, recombinations containing lateral mesoderm produced

mainly posterior epithelia. The mesoderm fate map shows that the lateral, but not anterior or dorsal, mesoderm is fated to produce smooth muscle so the large difference in fate between recombinations with lateral or dorsal mesoderm could be caused by the presence or absence of smooth muscle. Therefore, the signals causing the respecification may originate from the smooth muscle once it forms rather than the early mesoderm. The smooth muscle fate map means that future recombination studies to investigate the effect of mesoderm on endoderm development can be carried out with a knowledge of which parts of the mesoderm form smooth muscle in normal development.

In summary, this smooth muscle fate map shows that the rudiments of the smooth muscle are not in accord with the corresponding rudiments in the endoderm. This fate map should be useful for future studies into the specification of the smooth muscle and endoderm.

CHAPTER 7 MORPHOGENESIS OF THE GUT

7.1 INTRODUCTION

As well as regional specification, the development of an organ system also involves morphogenesis: that is, how the shape of the finished organ is formed from the cells of the early embryo. In several systems, cell rearrangements have been shown to be important in morphogenesis (discussed in chapter 1.10). However, prior to this study little was known about the morphogenesis of the endoderm and there were several important questions that had not been answered (see chapter 1.10). It was not known how the elongation of the endoderm is accomplished or how the thin dorsal layer and thick ventral layers of endoderm produce the single layer of cells that form the gut epithelium. Finally, it was not known whether the archenteron cavity of the neurula really gives rise to the gut cavity of the later tadpole.

In order to investigate the morphogenetic movements associated with gut formation two approaches have been used. The first approach used the cell lineage label DiI to specifically label four regions of endodermal cells. The regions labelled were; the dorsal roof of the archenteron, the floor of the archenteron, the middle endoderm that lies between the floor of the archenteron and the ventral endoderm and finally the very ventral endoderm that lies next to the mesoderm. The DiI labelling showed that these cells from these regions were incorporated into the gut epithelia and DiI/FDA double labelling was then used to show how cells in these different positions move relative to each other. This showed that radial intercalation occurred during formation of the epithelium and elongation of the gut. The second approach labelled the cells lining the entire surface of the archenteron with biotin. This allowed the cells of the embryonic cavity to be followed during development and showed that the archenteron almost closed before the definitive gut cavity opened from the remnant of the archenteron. Based on the results from these two approaches, a new model is presented that explains the morphogenesis of the gut epithelium.

7.2 RADIAL INTERCALATION PRODUCES THE GUT EPITHELIUM

At neurula stages the *Xenopus* endoderm consists of a single layer of dorsal cells and many layers of ventral cells (Fig. 7.1A). At stage 39/40 the archenteron lumen in the trunk region has become a very narrow cavity and often appears completely occluded (Fig. 7.1B). By stage 45 the endoderm has undergone massive elongation and formed the single layered epithelium that surrounds the gut cavity (Fig. 7.1C). Measurement of the pharynx and dissected gut from embryos showed that between stage 14 and stage 45 the endoderm increased in length approximately 5 times. From stage 41 to stage 45, when the gut is transformed from a straight tube to a coiled tube, the gut increases in length approximately 3.5 times. It is not clear how this transformation from the short, many layered embryonic endoderm to the long, single layered gut epithelia takes place.

A crucial question of gut formation is whether cells from each of the four positions shown in Figure 7.1A are incorporated into the gut epithelium. If the dorsal, floor, middle and ventral cells are all incorporated, then massive cell rearrangement must be occurring. In order to establish if these positions are incorporated, each position was labelled with the cell lineage label DiI. The embryos were then left to develop to the correct stage and the position of the label scored in sections or in dissected guts (see Materials and Methods).

In order to label the floor of the archenteron a piece was cut from the middle dorsal position to expose the floor of the archenteron. The floor was then labelled with DiI (see Materials and Methods) and the dorsal piece replaced and left to heal. In labelled embryos fixed at stage 14 a small clump of labelled cells can be seen in the middle of the archenteron floor (Fig. 7.1D, arrow). In embryos fixed at this stage the archenteron is shrunken because of the grafting but the label is clearly on the floor of the archenteron (Fig. 7.1D, insert). At stage 39/40, when the archenteron has narrowed, the labelled floor cells were still found in a quite dorsal position (Fig 7.1E) but had become spread out over a short stretch of the anterior/posterior axis. If a clear

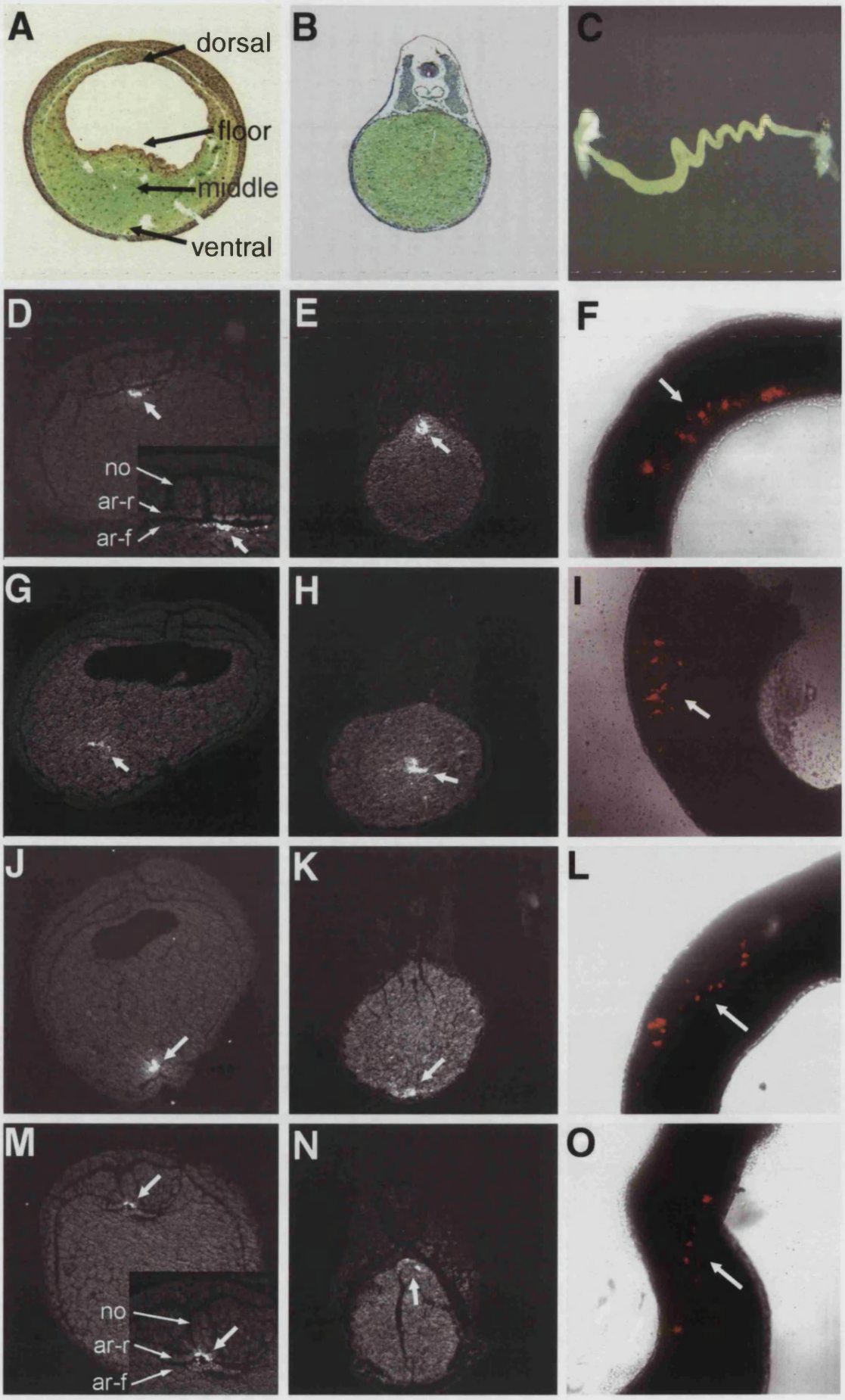
archenteron cavity was present the label was normally found abutting the ventral side of the lumen although occasionally a labelled cell was seen slightly more ventrally, separated from the lumen. At stage 45, once the intestinal epithelium has formed, the labelled cells were found incorporated into the intestinal epithelium (Fig. 7.1F).

In order to label the middle endodermal cells that lie between the floor of the archenteron and the very ventral endoderm a piece of tissue was cut from the mid ventral position (containing ectoderm, mesoderm and several layers of endoderm) and the exposed middle endoderm labelled with DiI. The piece of tissue was then replaced and the embryo left to heal. The labelled middle endoderm cells can be clearly seen at stage 14 (Fig. 7.1G). The middle cells remained in a middle position at stage 39/40 (Fig. 7.1H) and were incorporated into the gut at stage 45 (Fig. 7.1I). This shows that the yolky cells of the archenteron floor and middle endoderm do not disintegrate during development but become an integral part of the gut epithelium.

The extreme ventral and dorsal endoderm was then labelled to show how these regions move relative to the floor and middle cells. In order to label the mid-ventral endoderm a piece of ectoderm and mesoderm was cut from the mid ventral position of a stage 14 embryo exposing the endoderm. The endoderm was labelled with DiI and the piece replaced and the embryo left to heal. The ventral-most cells of the endoderm labelled at stage 14 (Fig 7.1J) were found at stage 39/40 to have maintained their ventral position next to the mesoderm (Fig. 7.1K) and at stage 45 were found incorporated into the intestinal epithelium (Fig 6L). The proximal/distal position of the label in the intestine was consistent with the FDA fate mapping.

In order to label the dorsal endoderm a piece was cut from the middle dorsal position, turned over, and the exposed archenteron roof was labelled with DiI. The piece of tissue was then replaced and left to heal. The archenteron roof appears partially collapsed in embryos fixed as stage 14 but despite this the labelled cells are clearly in the roof and not the floor of the archenteron (Fig 7.1M). These cells were still dorsal

Figure 7.1. DiI labelling of the *Xenopus* endoderm. A, section of control stage 14 embryo. The four labelling positions are marked with arrows. B, section of control stage 40 embryo. C, wholemount stage 45 control gut. D, section of floor DiI labelling at stage 14 (archenteron is shrunken because of the replacement of the roof). Insert shows high magnification view. E, section of floor DiI labelling visualised at stage 39. F, Floor DiI labelling visualised in a stage 45 wholemount. G, section of middle labelling at stage 14. H, section of middle DiI labelling visualised at stage 39. I, middle DiI labelling visualised in a stage 45 wholemount. J, section of ventral DiI labelling at stage 14. K, section of ventral DiI labelling visualised at stage 39. L, ventral DiI labelling visualised in a stage 45 wholemount. M, section of dorsal DiI labelling at stage 14. Insert shows high magnification view. N, section of dorsal DiI labelling visualised at stage 39. O, dorsal DiI labelling visualised in a stage 45 wholemount. Arrows highlight DiI label. Abbreviations; no, notochord, ar-r, archenteron roof, ar-f, archenteron floor.


A
dorsal
floor
middle
ventral
B
C
D
no
ar-r
ar-f
E
F
G
H
I
J
K
L
M
no
ar-r
ar-f
N
O

at stage 39/40 (Fig. 7.1N) and were also incorporated into the epithelium at stage 45 (Fig. 7.1O). As expected from the FDA fate mapping the dorsal endoderm labelled more proximal small intestine than the ventral endoderm, and often labelled the pancreas as well.

In each case (dorsal, floor, middle and ventral) a small coherent patch of labelled cells spread out to form a proximal/distal strip of labelled cells interspersed with unlabelled ones. As cells from each position became incorporated into the single layered epithelium it was inferred that, during formation of the gut epithelium radial intercalation of endodermal cells must be occurring.

To prove that radial intercalation was occurring and to establish in which directions it was occurring double labelling was carried out. In order to label the floor and ventral endoderm a shallow graft (containing ectoderm, mesoderm and approximately 1/2 layers of endoderm cells) was cut from an FDA labelled donor embryo and used to replace the equivalent piece from an unlabelled host. The embryo was left to heal and then the floor was labelled with DiI as described above. Surprisingly, this showed that the floor cells and the ventral cells ended up on opposite sides of the gut tube at stage 45 (Fig. 7.2B).

This suggested that the floor and dorsal cells end up on the same side of the gut tube. In order to test this the floor and dorsal endoderm were labelled. A piece of tissue was cut from the dorsal roof (equivalent to middle and posterior dorsal locations) of a host embryo and the archenteron floor labelled with DiI as above. A graft from an FDA labelled donor embryo was then used to replace the dorsal roof and the embryo left to heal. The dorsal and floor cells were later found intermingled on the same side of the gut tube (Fig. 7.2C) showing that the floor cells undergo radial intercalation with the dorsal cells as the epithelium is forming.

The final double labelling experiment was to label the middle endoderm with DiI and ventral endoderm with FDA. The middle endoderm was labelled with DiI as above. The piece of ventral tissue containing ectoderm, mesoderm and endoderm that was

removed to expose the middle endoderm was then replaced with the equivalent piece of tissue from an FDA labelled embryo. At stage 45 these cells were found intermingled on the same side of the gut tube (Fig. 7.2D). The DiI labelled cells were also found just lateral to the FDA labelled cells (Fig. 7.2D, a couple of DiI labelled cells can be seen to the left of the gut in a more lateral position). This demonstrated that the middle and ventral cells undergo radial intercalation to end up on the same side of the gut tube.

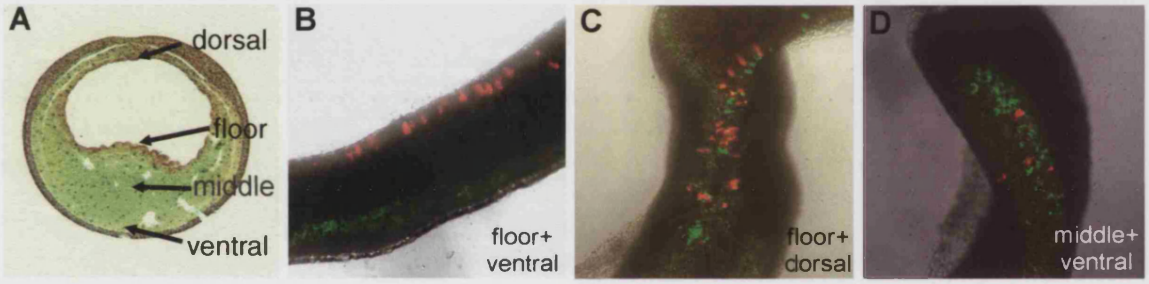
The DiI and DiI/FDA double labelling shows that radial intercalation occurs between the floor and dorsal cells and the middle and ventral cells. This produces the single layered epithelia from the many layered endoderm. Radial intercalation would increase the surface area of the tissue (Keller, 1980; Keller, 1987; Wolpert, 1998), so it could also be driving the elongation of the gut tube (see chapter 7.5).

7.3 THE ARCHENTERON CAVITY AND GUT LUMEN

The double labelling demonstrates that the cells on the dorsal roof and floor of the archenteron end up on one side of the gut cavity while the cells of the middle and ventral endoderm end up on the other side. This result is consistent with the archenteron closing and the definitive gut cavity opening up *de novo* in a position that is ventral and completely separate from the remaining archenteron cavity. However the results could also be explained by the archenteron narrowing and then widening to split the ventral endoderm and form the definitive gut cavity. To distinguish between these two possibilities it is necessary to label the cells surrounding the archenteron and to follow the progress of the cavity during development.

Sulfo-NHS-LC-biotin has previously been used to label the superficial cells of pre-gastrulation *Xenopus* embryos with biotin (Minsuk and Keller, 1997; Muller and Hausen, 1995). Streptavidin binds very tightly to biotin so the labelled cells can be detected by using streptavidin conjugated to a suitable marker such as alkaline phosphatase. This means that biotin labelling can be used to follow the superficial

Figure 7.2. DiI/FDA double labelling of the *Xenopus* endoderm. A, section of stage 14 control embryo. The four labelling positions are marked with arrows. B, floor DiI and ventral FDA double labelling visualised in a stage 45 wholemount. C, floor DiI and dorsal FDA double labelling visualised in a stage 45 wholemount. D, middle DiI and ventral FDA double labelling visualised in a stage 45 wholemount.


A
dorsal
floor
middle
ventral
B
floor+
ventral
C
floor+
dorsal
D
middle+
ventral

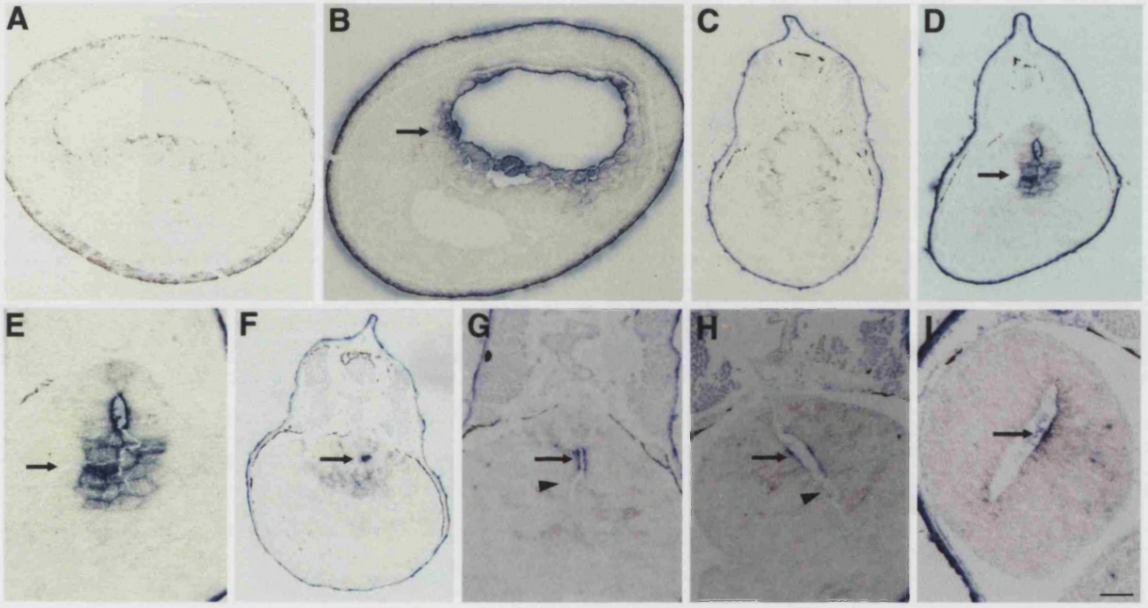
layer during development. After gastrulation these superficial cells will give rise to the cells that line the archenteron cavity (Keller, 1975; Minsuk and Keller, 1997; Smith and Malacinski, 1983), so this method can be used to label the archenteron cells.

Early stage 10 embryos were placed in the biotin labelling solution for 5 minutes after which the reaction was quenched and the embryos left to develop. At the correct stage the embryos were fixed, sectioned and the biotin labelled cells detected using alkaline phosphatase conjugated to streptavidin (see Materials and Methods).

The biotin treatment gave good strong labelling of the archenteron cells at stage 14 (Fig. 7.3B, arrow) with no labelling in the rest of the endoderm or in untreated control embryos (Fig. 7.3A). At later stages endogenous staining was seen in the epidermis of untreated control embryos but not in the gut (Fig. 7.3C). By stage 38 the archenteron had closed to a narrow slit through much of the gut (Fig. 7.3D). At this stage a number of labelled cells can be seen to have separated from the residual archenteron cavity and now lie ventral to it (Fig. 7.3D+E, arrow). The number of these cells varies slightly between individuals and along the anterior/posterior axis with fewer isolated cells seen in the posterior gut. This shows that closure of the archenteron occurs by cells becoming separated from the archenteron. At stage 40 the archenteron has narrowed further so that it often appears to have completely closed. Despite this a small ring of the biotin labelled cells was always clearly visible (Fig. 7.3F, arrow). The cells that separated from the closing cavity, clearly visible at stage 38, appear to have lost their label by stage 40. This is probably because as these cells leave the epithelium they lose their polarisation, resulting in a consequent increase in the turnover of cell surface proteins which would remove the biotin label.

In the more posterior regions of the stage 40 intestine the archenteron cavity does not close up so much (Fig. 7.3G) and it appears that in certain regions it has started to widen in a ventral direction. This gives rise to a cavity with a region of labelled cells (arrow) and one of unlabelled cells (arrow head). At stage 42 the archenteron in the

Figure 7.3. The archenteron cavity during development. The superficial cell layer that gives rise to the cells lining the archenteron was labelled at stage 9/10. The labelled cells lining the archenteron could then be followed during development. A, unlabelled stage 14 embryo. B, biotin labelled cells at stage 14 (arrow). C, unlabelled stage 38 embryo showing endogenous signal in the epidermis but not the gut. D+E, biotin labelled cells (arrow) at stage 38 showing partial closure of archenteron. F, biotin labelled cells (arrow) at stage 40 showing small persistent archenteron. The label is lost from the internalised cells. G, biotin labelled cells in the posterior gut of a stage 40 embryo showing new cavity opening (arrow-biotin, arrowhead-unlabelled). H, biotin labelled cells in the posterior gut at stage 42 showing cavity is only partially labelled (arrow-biotin, arrowhead-unlabelled). I, biotin labelled cells (arrow) in stage 44 gut showing partial labelling of new cavity. Scale bar 100 μm except E, G, H and I where it is 50 μm.


A
B
C
D
E
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H
I

middle of the gut is still very small while in the posterior of the gut the cavity has continued to widen (Fig. 7.3H). Once the gut cavity has formed the biotin labelling can be seen in one small segment of the circumference (Fig. 7.3I).

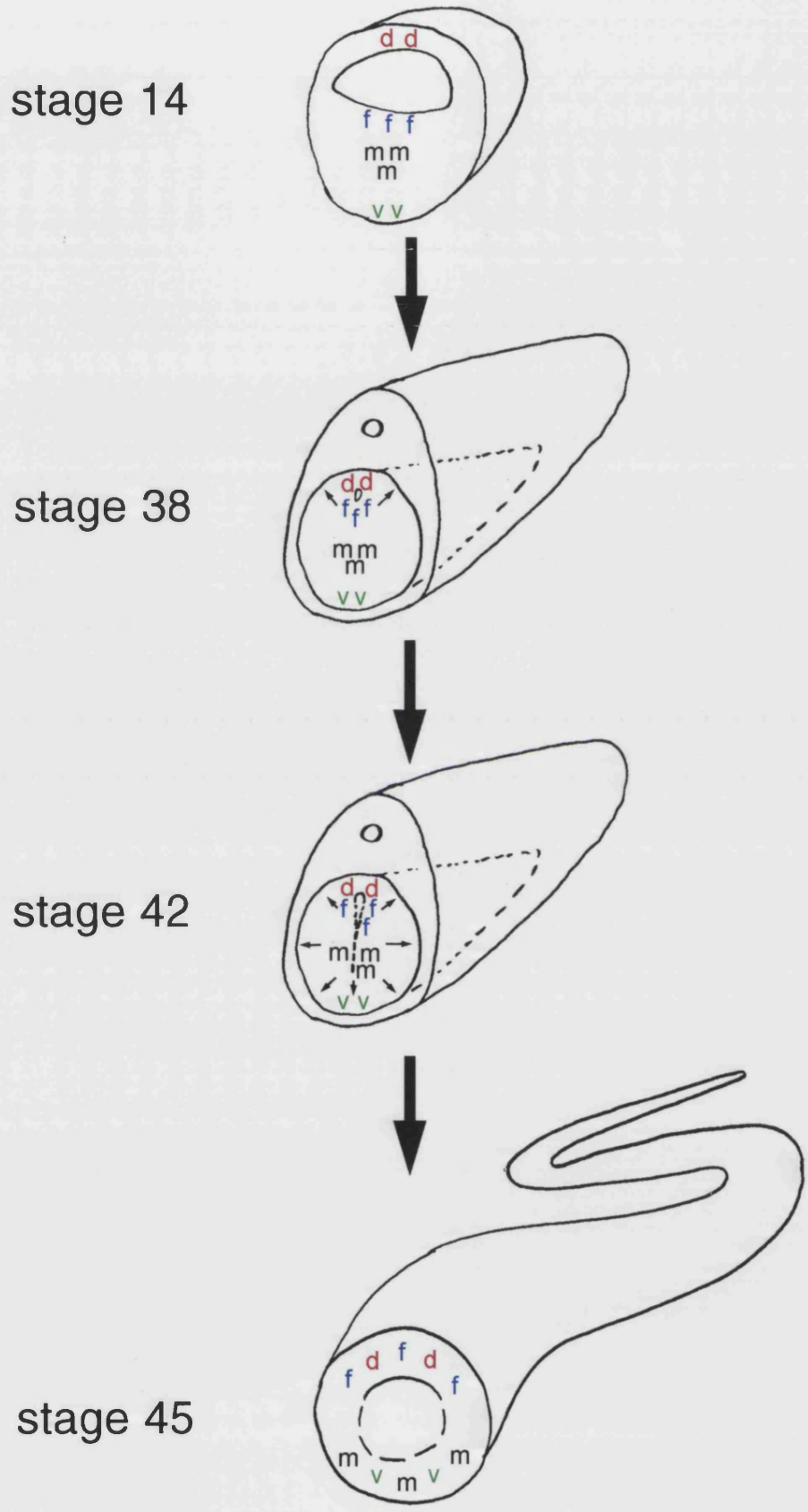
This biotin labelling study shows that the archenteron narrows to a very small cavity and some of its lining cells become dispersed into the ventral endoderm. Later the definitive gut cavity is formed by enlargement of a split originating from the archenteron remnant. This split divides the endoderm down the middle and the radial intercalation, demonstrated by the DiI labelling, transforms the short thick undifferentiated gut tube into the long thin epithelium.

7.4 A MODEL FOR MORPHOGENESIS OF THE GUT

Based on the combined evidence from the endoderm fate map, the DiI labelling and the biotin surface labelling experiments a model has been proposed explaining the morphogenesis of the gut (Fig. 7.4). From neurula stages to stage 38 the archenteron gradually closes. This is achieved by cells leaving the cavity lining to be incorporated into the deeper endoderm. These movements leave the original dorsal (d), floor (f), middle (m) and ventral (v) cell groups in similar relative positions. From stage 38 to stage 45 the remains of the archenteron expand to split the ventral endoderm. This gives rise to the definitive gut cavity which contains the cells of the original archenteron and the more ventral endoderm.

The DiI labelling shows that the floor endoderm intercalates with the dorsal endoderm and the middle endoderm intercalates with the ventral/lateral endoderm. This radial intercalation causes the opening of the gut cavity from the remains of the archenteron and would increase the surface area of the gut accounting for part of the elongation of the gut.

Figure 7.4. Morphogenesis of the gut epithelium. From stage 14 to stage 38 the embryonic archenteron narrows to a small cavity. During this time the dorsal (d), floor (f), middle (m) and ventral (v) cell populations retain their relative positions. After stage 38 the gut cavity starts to open from the remains of the archenteron to split the ventral endoderm. This produces a cavity, part of which originated from the archenteron (solid line), and part of which is the new cavity (dashed line). By stage 45 the gut cavity is fully open. The splitting of the ventral endoderm places the cells of the archenteron (f + d) on one side of the cavity and the middle (m) and ventral endoderm cells (v) on the other side. Radial intercalation may drive both the opening of the gut cavity and the elongation of the gut.


stage 14
d d
f f f
m m
m
v v
stage 38
d d
f f f
m m
m
v v
stage 42
d d
f f
f
m m
m
v v
stage 45
d f d
f f
m m
v m v

7.5 DISCUSSION

Xenopus extraembryonic endoderm. It has been proposed that some of the large yolky cells of the *Xenopus* endoderm may be extraembryonic (see chapter 5.6). The large yolky ventral endoderm cells have also been reported to disintegrate during development (Mathews and Schoenwolf, 1998; Nieuwkoop and Faber, 1967). This also implies that these cells are extraembryonic, but in both cases there is no evidence that these cells are extraembryonic except that they are big and yolky. The endoderm fate map showed that there is no large region of extraembryonic tissue in the *Xenopus* endoderm. However, this approach did not address whether certain regions within the grafts, such as the floor of the archenteron, are extraembryonic. The DiI labelling showed that dorsal, floor, middle and ventral endoderm cells all contributed to the intestinal epithelium. This shows that the large yolky ventral endoderm cells are not all extraembryonic. It does remain possible that a small percentage of the cells labelled with DiI are extraembryonic. However, the only argument for them being extraembryonic is that they are big and yolky, and the DiI labelling shows that the big and yolky ventral endoderm cells do contribute to the epithelium. Therefore, the data from this work strongly suggests that there is no extraembryonic endoderm in *Xenopus*.

Active or passive intercalation. The DiI labelling demonstrated that radial intercalation occurs in the endoderm. Cell rearrangements can be an active, force generating process, or a passive event that responds to external forces (Keller, 1987). The intercalation that takes place during gastrulation in *Xenopus* occurs autonomously in explanted tissues. This shows that the cell intercalation is an active process that drives the changes in shape that occur during gastrulation (Keller, 1984; Keller and Danilchik, 1988; Keller *et al.*, 1985; Shih and Keller, 1992a; Shih and Keller, 1992b).

The radial intercalation in the gut could be an active process that drives the elongation of the gut and the opening of the gut cavity. Alternatively the intercalation could be a passive process that causes the opening of the gut cavity and elongation of

the gut in response to a force applied from other regions of the embryo. This work does not distinguish between these two possibilities. However, the DiI labelling shows that the radial intercalation occurs between stage 40 and stage 45 when the gut is transformed from a straight tube to a complex coiled structure and elongates approximately 3.5 times. The elongation of the endoderm that occurs once the gut has started to coil can not be linked to the elongation of the body axis, so it must occur by a separate active process. It seems likely that this active process is the radial intercalation of the endoderm and it drives both the elongation of the gut and the opening of the gut cavity.

Why close the archenteron cavity. The biotin labelling showed that the archenteron cavity almost closed before the new gut cavity opened up from the remnant of the archenteron. One question that arises is why the archenteron cavity closes before opening up again to produce the new gut cavity. A possible answer to this is that if the radial intercalation occurred around the archenteron cavity it would all take place on the ventral side. This would produce extremely unequal elongation between the dorsal and ventral sides. The new cavity that opens up from the remains of the archenteron splits the ventral endoderm down the middle. This would produce symmetric intercalation and elongation, thus overcoming this problem.

Interestingly the gut starts to coil at the same time as the intercalation is occurring. This raises the possibility that, even though the intercalation is much more even than it would be if it occurred around the archenteron, there could be slightly more intercalation on the ventral side of the gut. This unequal intercalation would produce slightly more elongation of the ventral side causing the gut to coil up. Therefore, it is possible that the radial intercalation may be driving the coiling of the gut as well as the opening of the gut cavity and the elongation of the gut.

In summary, the results from a number of labelling techniques have been used to produce a model that explains the transition from early endoderm to gut epithelium.

CHAPTER 8 FINAL DISCUSSION AND FUTURE PROSPECTS

8.1 A THOROUGH STUDY OF THE ENDODERM-DERIVED ORGANS

The advantages of *Xenopus* embryos mean that they offer numerous opportunities for the study of endoderm development. However, prior to this work, a problem with studying the endoderm-derived gut was that the complexity of the coiled gut made it very difficult to identify organs and to distinguish between normal and abnormal development of the gut. To overcome this problem a thorough study of the organs of the gut was carried out which allows any organ of the gut seen in a section to be identified and the position of that organ relative to the rest of the gut established.

To complement this work the anatomy of wholemount guts has also been described. The wholemount preparation offers the advantages of being able to quickly and easily visualise the organs of the gut and to check for gene expression in the gut using wholemount *in situ* hybridisation. This description, which has been published (Chalmers and Slack, 1998), was an essential prerequisite to the rest of this study and should be useful for future studies into gut development.

8.2 COMPARISON WITH THE GUT OF OTHER VERTEBRATES

An important consideration if the tadpole gut is to be considered a good model system for the study of gut formation, is that it should be similar to the gut of other vertebrates. At a gross level the tadpole gut was found to have the same organs as other vertebrates. The cell types of the gut were also shown to be generally similar to the developing vertebrate foetal gut although there were some differences. One interesting difference between the tadpole gut and the gut of other vertebrates is that the development of the intestinal epithelium and smooth muscle seems delayed in the tadpole gut. This delay in development is maintained until metamorphosis when the

intestinal structure becomes more similar to other vertebrates. Paradoxically this difference in intestinal development may actually prove useful for the study of intestine development. It could be that some processes of intestine development are separated to the early or late phases of development which might make it easier to distinguish between the role of individual processes in the development and differentiation of the intestine.

The patterns of gene expression in the tadpole gut were also found to be similar to that of other vertebrates. It had been previously shown that *IFABP* and *Xlhbox8* show similar expression patterns to their vertebrate homologues. This work showed that *Xcad1*, *Xcad2* and *Xsox2* also show similar expression patterns in the tadpole gut to the vertebrate gut. This conserved expression strongly suggests that the mechanisms of gut development have also been conserved. Further evidence that the tadpole gut has similar mechanisms of development to other vertebrates was shown by the fact that, in transgenic *Xenopus,* mammalian promoters can drive the correct pattern of expression in the tadpole gut (Beck and Slack, 1999). The similarities in organs, cell types, gene expression and promoter function suggest that the study of the development of the tadpole gut is likely to be relevant to the development of the gut of other vertebrates.

8.3 GENE EXPRESSION IN THE TADPOLE GUT

This study showed that *Xsox2, Xcad1* and *Xcad2* are expressed in the *Xenopus* tadpole gut epithelium. However, one limitation of this work was that it was not possible to be certain whether these genes were also expressed in the closely associated smooth muscle (discussed in chapter 4.3). A useful piece of future work would be to confirm if these genes are expressed in the smooth muscle. This could probably be achieved by carrying out *in situs* to sections, rather than doing *in situs* to wholemounts and then sectioning them.

Once this limitation has been overcome, *Xsox2, Xcad1* and *Xcad2* will be useful molecular markers for the gut that could be used along with the previously described endodermal markers in future studies of endoderm development. It would also be

interesting to try and establish what role these genes play in gut development. In order to try and establish their function, they could be expressed in different parts of the gut, using the mammalian gut promoters. It may also be possible to try and block their function by using these promoters to drive expression of antimorphic versions of these transcription factors. This approach is attractive as it will not affect early development. This would solve the problem of early lethality that sometimes occurs when trying to study the role of genes in gut development in knockout mice. For example, *cdx2* homozygous mutant mice die early in development before an effect on the development of the intestine can be established (Chawengsaksophak *et al.*, 1997). Establishing the role of these factors in gut development would be an important advance.

8.4 FATE MAPS AND ENDODERM SPECIFICATION

The endoderm fate map that has been produced in this study shows which regions of the endoderm give rise to the epithelia of which organs of the gut and respiratory system. A second fate map has also been produced which shows where the smooth muscle which covers the endoderm-derived epithelia originates from. Comparison of the two fate maps shows that for most organs of the gut the two sets of rudiments do not overlie each other in the early embryo, so must move into accord in later development.

There is evidence that the early endoderm contains some pattern (discussed in chapter 1.7). One possible mechanism for producing this pattern was that there could be early as well as late signals from the cells that will form the gut mesenchyme. However, the fate maps show that early in development the layers are not in accord, which rules out this possibility. Therefore, the early patterning of the endoderm must be caused by an alternative and unknown mechanism.

Now that an accurate fate map has been produced future work should be able to take explants of the *Xenopus* endoderm to establish whether or not the neurula endoderm contains pattern. If the neurula endoderm is found to contain a lot of pattern then some of the molecules that have been shown to play a role in the formation of the

endoderm (see chapter 1.3) could be investigated to establish if they are also important in the patterning of the endoderm. If the endoderm is found to have little pattern, the role of the mesoderm in the patterning of the endoderm could be investigated by recombining explants of endoderm with explanted mesoderm. The fate map for the smooth muscle means that the recombinations can now be done with the knowledge of whether or not the piece of mesoderm is fated to form smooth muscle. This should make it easier to distinguish between the effects of the early mesoderm and the effects of smooth muscle that develops from the mesoderm, on the development of the endoderm.

8.5 FATE MAPS AND SMOOTH MUSCLE SPECIFICATION

The smooth muscle and connective tissue form a crucial part of the digestive tract but comparatively little is known about how these tissues develop (McHugh, 1996). However, It has been shown that signals from the epithelia play a role in the development of the smooth muscle (Kedinger *et al.*, 1990; Takahashi *et al.*, 1998). This raises the possibility that as well as the mesenchyme signaling to the epithelia, the epithelia may also signal to the mesenchyme.

The smooth muscle fate map presented here means that it is now possible to explant the regions of the mesoderm that are fated to form smooth muscle to establish if the neurula stage mesoderm is already specified to form smooth muscle. Endoderm could also be explanted and recombined with the mesoderm to investigate the role of signals from the endoderm on the development of the smooth muscle.

In summary the two fate maps, which have been submitted for publication (Chalmers and Slack, 1999), represent a useful resource for studies into the specification of the endoderm and smooth muscle and for investigations into the role that each tissue plays in the development of the other.

8.6 EXTRAEMBRYONIC ENDODERM IN *XENOPUS*

The results from the endoderm fate map and DiI labelling strongly suggests that there are no regions of extraembryonic endoderm in *Xenopus*. However, it is important to stress that this data does not address whether, as has been suggested (see Beddington and Robertson, 1999; Jones *et al.*, 1999), the *Xenopus* endoderm has equivalent signalling functions to the mammalian extraembryonic endoderm. What this data shows is that although the *Xenopus* endoderm may have equivalent function to the mammalian extraembryonic endoderm it is not actually extraembryonic.

8.7 MORPHOGENESIS OF THE GUT

Before this work there were several important questions concerning the morphogenesis of the endoderm that were not understood. One of these unanswered questions was how the single layered epithelia formed from the many layered endoderm. The DiI labelling of the endoderm showed that the cells of the middle and floor endoderm were incorporated into the forming intestinal epithelium by radial intercalation. Radial intercalation not only explains how the epithelia forms, but as it would also cause an increase in area of the tissue, it also at least partially explains how the elongation of the endoderm is achieved.

The biotin labelling of the cells lining the archenteron explained another aspect of gut morphogenesis which was whether or not the archenteron cavity gives rise to the gut cavity. The archenteron cavity was shown to narrow and almost close before the definitive gut cavity opens from the remnant of the archenteron cavity. The results from the DiI labelling and the biotin labelling, which have been submitted for publication with the fate mapping work (Chalmers and Slack, 1999), were combined to produce a model explaining the morphogenesis of the gut epithelium.

In the future, it would be interesting to try and establish whether the radial intercalation that occurs during gut formation is a passive or an active event. The cell rearrangements that occur in the marginal zone during gastrulation have been shown to be an active process. Further observations of explanted marginal zones suggested a

possible driving force for these rearrangements. The cells that undergo radial and mediolateral intercalation in the marginal zone were found to extend cellular processes prior to the intercalation occurring (Keller, 1980; Shih and Keller, 1992a; Shih and Keller, 1992b). The processes are extended in the plane of the intercalation and attach to adjacent cells. The cells then apply traction on their neighbours which causes the intercalating cell to initially elongate and then intercalate.

Future work on the morphogenesis of the gut could examine the endodermal cells that were shown here to undergo radial intercalation to see if these cells also extend cellular processes during intercalation. If the cells were found to extend processes then it would be good evidence that the intercalation is an active process, which drives the opening of the gut cavity, formation of the epithelium and elongation of the gut. An alternative method to try and establish if the intercalation is an active process would be to dissect the endoderm from the embryo and see if, like the marginal zone, it elongates autonomously in culture. Future work should also look at other factors, apart from cell rearrangements, that could play a role in the morphogenesis of the endoderm. For example, cell division or apoptosis may be important in the morphogenesis of the endoderm.

This work represents a valuable start in the study of the development of the *Xenopus* tadpole gut and respiratory system. Hopefully, the future work suggested here will build on the results from this study and provide further insights into the mechanisms responsible for the development of the endodermal organs.

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